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Technical specifications for a European baseline survey of norovirus in oysters

European Food Safety Authority (EFSA)

Abstract

The European Commission requested scientific technical assistance in the preparation of a survey protocol for a European Union (EU) coordinated monitoring programme on the prevalence of norovirus (NoV) in raw oysters. The objective of the survey is to estimate the European prevalence of norovirus-contaminated oysters at production areas and batches of oysters at dispatch centres, with a 95% level of confidence and a level of precision of 5% considering an expected prevalence of 50%. The survey protocol defines the target population, the sample size for the survey, sample collection requirements, the analytical method for the quantification of NoV copy number (genotype I and genotype II), the data reporting requirements and the plan of analysis. The sample unit in production areas is a classified production area actively growing commercial oysters (whether harvesting or not is occurring) and for dispatch centres is a quantity of live oysters which are being packed and labelled with an Identification Mark. Based on a multistage sampling scheme, 1,026 samples from 171 production areas and 1,182 samples from 197 dispatch centres should be taken annually in Europe. To reduce the probability of surveying an atypical year, the survey is to be repeated for a second year. The samples are to be analysed according to the method specification developed by the European Union Reference Laboratory (EURL) expert working group, which is compliant with ISO/DIS 15216-1. Generalised linear models will be used to estimate proportion (with 95% confidence intervals) of sample units with NoV contamination for the following thresholds: < limit of quantification (LOQ), 100, 200, 500, 1,000, 5,000, 10,000 and > 10,000 copies/g. The necessary data to be reported by the sampler and the laboratory to support this analysis is presented in two data models. The results of the survey should be reported using the EFSA data collection framework.

Keywords: norovirus, oyster, survey, production area, dispatch centre

Requestor: European Commission

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Table of contents

1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Noroviruses (NoV) are known to cause 'winter-vomiting disease' or 'stomach-flu' referring to their rapid spread in human populations especially during winter months. They are transmitted primarily through the faecal-oral route, either by consumption of contaminated food or water, or by spreading directly from person to person. Many different food items have been associated with NoV outbreaks. Raspberries and oysters have caused several national and international outbreaks.

Bivalve molluscs are a well-documented source of noroviral infection since they have the ability to accumulate and concentrate NoV particles by filtration of water contaminated with faeces. NoV associated with point source human faecal pollution (e.g. discharges from sewage treatment works) is a persistent problem in coastal waters during the winter months leading to the contamination of bivalve mollusc production areas. Oysters contaminated with NoV pose a particular risk to human health since they are routinely consumed raw.

There is currently no threshold infectivity limit established for NoV as detected by polymerase chain reaction (PCR). The probability of becoming infected increases with the dose but depends also on the characteristics of the organism, the food matrix and the host factors.

Furthermore, the relationship between the number of infectious virus particles and the number of virus genome copies detected by quantitative PCR is not a constant, and it is important to realise that the infectious risk associated with low level positive oysters, as determined by real-time PCR, may be overestimated.

In accordance with Article 31 of Regulation (EC) No 178/2002, EFSA is requested to provide scientific and technical assistance on design related to an EU coordinated monitoring programme on the prevalence of NoV in raw oysters, and in particular to:

Prepare a survey protocol for the baseline survey of NoV contamination in oysters in the EU with the objective to:

a) Assess the proportion of EU classified production areas with NoV contamination. The survey should detect at least 1% of representative monitoring points within harvesting areas with > 1,000 total NoV PCR copies per gram with a level of confidence of 95%;

b) Assess the proportion of batches of final product at approved EU dispatch centres with NoV contamination. The survey should be able to detect batches with > 1,000 total NoV PCR copies per gram with a level of confidence of 95% and a level of precision of 5% and an expected prevalence of 50%.

1.2. Interpretation of the Terms of Reference

The working group agreed that the objective of the survey is to estimate the likelihood that a sample unit is contaminated with NoV. As a consequence, the working group proposed an interpretation of the survey objective for classified production areas as the estimation of prevalence of NoV in that population.

The baseline survey should assess the proportion of European Union (EU) classified oyster production areas with NoV contamination. The survey should estimate the prevalence of norovirus (> 1,000 total NoV PCR copies/g) in oysters collected from representative monitoring points within production areas with a level of confidence of 95% and a level of precision of 5% and considering an expected prevalence of 50%.

The estimation of prevalence based on viral copy number (> 1,000 total NoV PCR copies/g) is primarily included in the survey objectives to indicate that the survey requires the quantification of viral RNA to be reported rather than just the detection of viral RNA. **The final data analysis will present the proportion of contaminated production areas/batches of final product for a range of NoV copy number thresholds (below limit of quantification (LOQ), 100, 200, 500, 1,000, 5,000, 10,000 and greater than 10,000 copies/g)** in order to refine the analysis presented in EFSA BIOHAZ Panel, 2012.

An expected prevalence of 50% is specified in the objective as this represents the maximum variability and therefore the largest sample size. For thresholds based on a lower copy number than 1,000 where the expected prevalence could be greater than 50% this sample size is more than sufficient to achieve the required precision. In general, a higher level of precision is achieved if the prevalence at a specific copy number threshold is greater or lower than 50% (Bartlett et al 2001).

2. Data and Methodologies

2.1. Survey design

2.1.1. Survey objectives

Survey objectives can broadly be divided into two groups: estimation or inferential. Estimation objectives mainly involve production of quantitative and numerical descriptions (estimation) of relevant aspects of a target population, like the population mean or the population total, mean difference between two groups of the same population and proportion of the population with a trait of interest, etc. On the other hand, inferential objectives are about testing a particular hypothesis about the population of interest, examples include, testing that the population mean is greater (less) than a certain value, or that means of groups of the same population are not equal (Milanzi et al, 2015). For the baseline survey, we are seeking to estimate the likelihood that a sample unit is contaminated with NoV.

The first objective is to estimate the prevalence of norovirus-contaminated (> 1,000 total NoV PCR copies/gram) oyster production areas, at EU level, with a level of confidence of 95% and a level of precision of 5% and considering an expected prevalence of 50%

The second objective is to estimate the prevalence of norovirus-contaminated (> 1,000 total NoV PCR copies/gram) batches at approved dispatch centres, at EU level, with a level of confidence of 95% and a level of precision of 5% and considering an expected prevalence of 50%.

A clear definition of targeted population is important to determine the extent to which results from the survey can be generalised (Milanzi et al, 2015).

In Europe, oyster production is primarily comprised of two species: flat oysters (*Ostrea edulis*) and Pacific cupped oysters (*Crassostrea gigas*).[1] The production of Portuguese oysters (*Crassostrea angulata*) was reported in production areas in Spain and Portugal (information received from EU Member State (MS) Competent Authorities represented on Commission working group on live bivalve molluscs (LBM)). A study of artificial contamination of *O. edulis* and *C. gigas* with NoV at a range of concentrations concluded that there was no evidence that the performance of the detection method varied significantly between the two oyster species (CEFAS 2011). It is assumed that there will be no significant difference between the three species being produced in Europe. Therefore, the oyster species *O. edulis, C. gigas* and *C. angulata* are to be sampled in the survey. Only **live** oysters are to be sampled for the survey.

The terms of reference request both 'representative monitoring points within harvesting areas' and 'batches of final product from approved EU dispatch centres' to be included in the survey. Therefore, survey requires samples to be taken at two points in the food chain. Sampling in production areas is closer to the source of contamination whereas sampling oysters as they leave the dispatch centre is closer to the consumer exposure. The inclusion of both locations will ensure that suitable data is available to support a decision with regard to setting a microbiological criterion for NoV.

[1] http://ec.europa.eu/fisheries/cfp/aquaculture/aquaculture_methods/index_en.htm

Production areas

Harvesting areas include both production areas and relaying areas (EC, 2012). 'Production area' means any sea, estuarine or lagoon area, containing either natural beds of bivalve molluscs or sites used for the cultivation of bivalve molluscs, and from which live bivalve molluscs are taken. 'Relaying area' means any sea, estuarine or lagoon area with boundaries clearly marked and indicated by buoys, posts or any other fixed means, and used exclusively for the natural purification of live bivalve molluscs.[2]

The production areas from which harvesting of bivalve molluscs is authorised are divided into three classes based on monitoring results for the bacterial indicator organism *Escherichia coli*:

Class A areas: Areas from which molluscs may be collected for direct human consumption (less than or equal to 230 *E. coli*/100 g of flesh and intravalvular liquid in 80% of samples. The remaining 20% must not exceed 700 *E. coli*/100 g flesh and intravalvular liquid).[3]

Class B areas: Areas from which molluscs may be collected but may only be placed on the market for human consumption after treatment in a purification centre, after relaying or after an approved heat treatment (less than or equal to 4,600 *E. coli*/100 g of flesh and intravalvular liquid (in 90% of samples and no sample more than 46,000 *E. coli*/100 g of flesh and intravalvular liquid)). [4]

Class C areas: Areas from which molluscs may be collected but may only be placed on the market after relaying over a long period (as a general rule, at least 2 months) whether or not combined with purification or after an approved heat treatment (less than or equal to 46,000 *E. coli*/100 g flesh and intravalvular liquid).[4]

The classification status of a production area can vary with time and is dependent on the *E. coli* monitoring results. Full classification is based on results from an extensive number of sampling occasions to ensure that potential seasonal and annual variability has been fully covered. Seasonal classification is given when there is a clear seasonal trend over a number of seasons and, consequently, different classification categories apply for different seasons (EC, 2012). Class A, B and C production areas are to be included in the survey.

A representative sampling point is a specified geographical location from which samples are taken to represent either a single or several, wild bivalve mollusc beds or aquaculture sites. The representative sampling point should reflect the location at highest risk of faecal pollution within the classified area. For offshore areas (> 5 km from shore) not impacted by point discharges (according to the sanitary survey) random sampling points within the classified area may be used.

In order to ensure that a suitable sample of live oysters for laboratory analysis can be obtained, the target population is defined **as classified production areas actively growing commercial oysters (whether harvesting or not is occurring). Approved relaying areas are excluded** for logistic reasons**,** since they are only used for natural purification for limited periods in the year. A commercial oyster is defined as an oyster that is of sufficient maturity to enter the production chain for consumption.

Dispatch centres

'Dispatch centre' means any onshore or offshore establishment for the reception, conditioning, washing, cleaning, grading, wrapping and packaging of LBM fit for human consumption[2]. Dispatch centres are approved by the Competent Authority (CA) and assigned a unique approval number (Appendix D).

'Batch' means a group or set of identifiable products obtained from a given process under practically identical circumstances and produced in a given place within one defined production period[5]

[2] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin

[3] Commission Regulation (EU) 2015/2285 amending Annex II to Regulation (EC) No 854/2004 of the European Parliament and of the Council laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption as regards certain requirements for live bivalve molluscs, echinoderms, tunicates and marine gastropods and Annex I to Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs.

[4] Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption.

[5] Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs

For the purposes of the survey, a batch of final product means **a quantity of live oysters, which are being packed and labelled with an Identification Mark**. This quantity of oysters should have a single unique batch identifier in the dispatch centre internal traceability system.

In line with permitted activities under EU legislation, the following batches can be included for sampling:

- batches containing oysters from one or multiple production areas;
- batches containing oysters from European or third country production areas;
- batches containing class A, B or C oysters following, if necessary, purification, conditioning or relaying;
- batches containing oysters from existing batches, which are being reassembled into smaller or larger packages for individual customers.

Figure 1 is a generalised schematic illustrating relationship between production areas and dispatch centres, and the processes that can be applied to molluscs prior to placing on the market for consumption. Three processes can be applied to molluscs prior to consumption depending on the classification of the production area from which they were harvested.

Conditioning is the storage of LBM coming from class A production areas, purification centres or dispatch centres in tanks or any other installation containing clean seawater, or in natural sites, to remove sand, mud or slime, to preserve or to improve organoleptic qualities and to ensure that they are in a good state of vitality before wrapping or packaging. Conditioning is not performed in all dispatch centres.

Relaying is the transfer of LBM to sea, lagoon or estuarine areas for the time necessary to reduce contamination to make them fit for human consumption. This does not include the specific operation of transferring bivalve molluscs to areas more suitable for further growth or fattening.

Purification treatment is performed in tanks fed by clean seawater in which LBM are placed for the time necessary to reduce contamination to make them fit for human consumption

In some cases, the production chain can be more complex and there may be regional variations depending on local conditions and production practices.

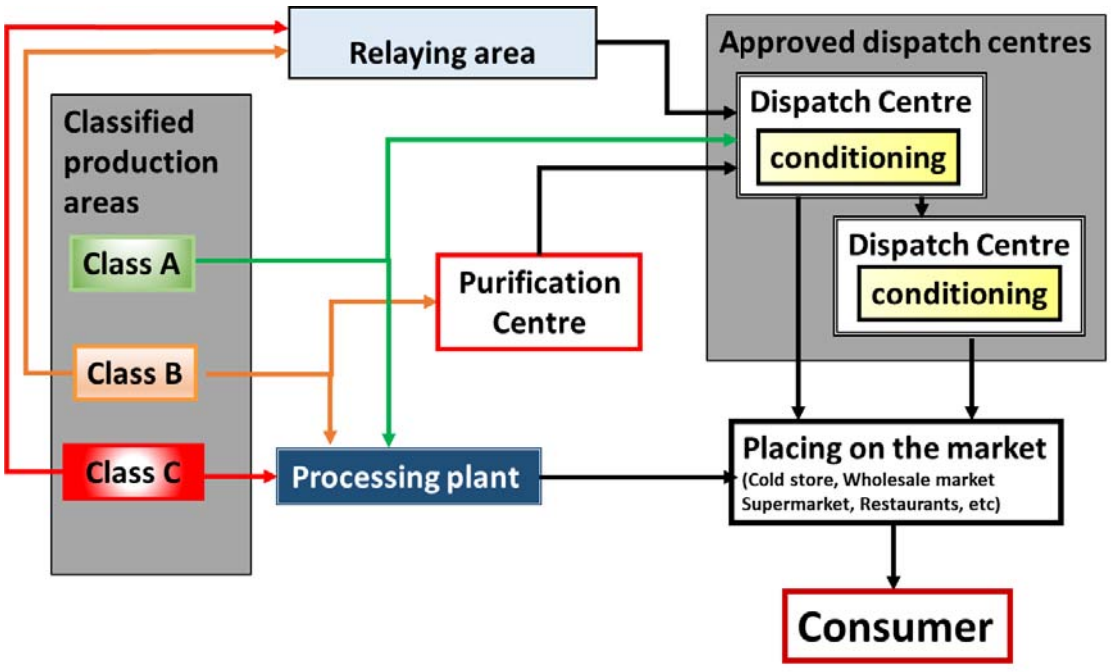

Figure 1: Schematic of the production chain for bivalve molluscs

2.1.2. Sampling frame

Representative sampling points within production areas

Regulation (EC) No 854/2004 specifies that the CA must fix the location and the boundaries of production areas for bivalve molluscs. In some cases, there may be more than one representative sampling point within a classified production area. For the baseline survey, a single representative sampling point within a production area should be selected for sampling the oysters. This should be the sampling point with highest levels of *E. coli* contamination based on the routine monitoring performed under Regulation (EC) No 854/2004 (see Section 2.2.1 for more details).

Information on classified oyster production areas was requested from the MSs by the European Union Reference Laboratory (EURL) and a summary is presented in Table 1. In May 2015, there were 422 classified production areas that had produced oysters in the last 12 months in 13 countries (12 MS plus Norway). In order to refine the sampling plan at MS level, the number of representative sampling points per production area, information on the current status of production areas and the availability of commercial size oysters throughout the year should be obtained prior to starting the survey. Figure 2 shows the population hierarchy considered in the survey design for sampling from production areas.

Batches of final product from approved EU dispatch centres

Approved dispatch centres apply Identification Marks at the point of packing and wrapping prior sending the batches to their customers. Information on approved dispatch centres was requested from the MS by the EURL and a summary is presented in Table 1. In May 2015, there were 2,325 approved dispatch centres that had packed oysters in the last 12 months in 13 countries (12 MS plus Norway). Prior to starting the survey, the operational status of approved dispatch centres within a MS should be confirmed. In addition, information on the batches produced monthly in the dispatch centres in 2014 should be obtained. If this information is not available, production volumes per dispatch centre in kilograms could be used as a proxy. This information would allow refinement of the sampling plan at MS level and adjustment of the timing of sampling around seasonal fluctuations in availability of oysters. Figure 3 shows the population hierarchy considered in the survey design for sampling of batches of final product from dispatch centres.

Table 1: Number of classified oyster production areas and number of approved dispatch centres packaging oysters (Summary of information received from Competent Authorities represented on Commission working group on LBM, May 2015)

	Production Areas				Dispatch Centres			
	Total (a)	Active	Not Active	Don't Know	Total	Active	Not Active	Don't Know
Croatia	6	5	1		9	9		
Denmark	6	6			5	5		
France	201	189	4	8(c)	2442	2015		427
Germany	6	1		5	3	2	1	
Greece	3	3			3	3		
Iceland	1		1		0			
Ireland	66	56	10		12	12		
Italy	37	18	17	2	161	110	47	4
Netherlands	11	11			34	33	1	
Norway	3	3			3	1	2	
Portugal	16	11		5	30	21	9	
Romania	1		1		0			
Spain	121	34(d)	263(d)	33(d)	70	70		
Sweden	10	6	4		5	3(b)	3(b)	
United Kingdom	80	79	1		46	41	5	
Total		**422**				**2,325**		

LBM: live bivalve molluscs
(a): Totals may vary depending on the number of species present in the production area
(b): Combined data for *C. gigas* and *O. edulis*
(c): Storage areas, in development, small production
(d): Combined data for *C. gigas, O. edulis* and *C. angulata*

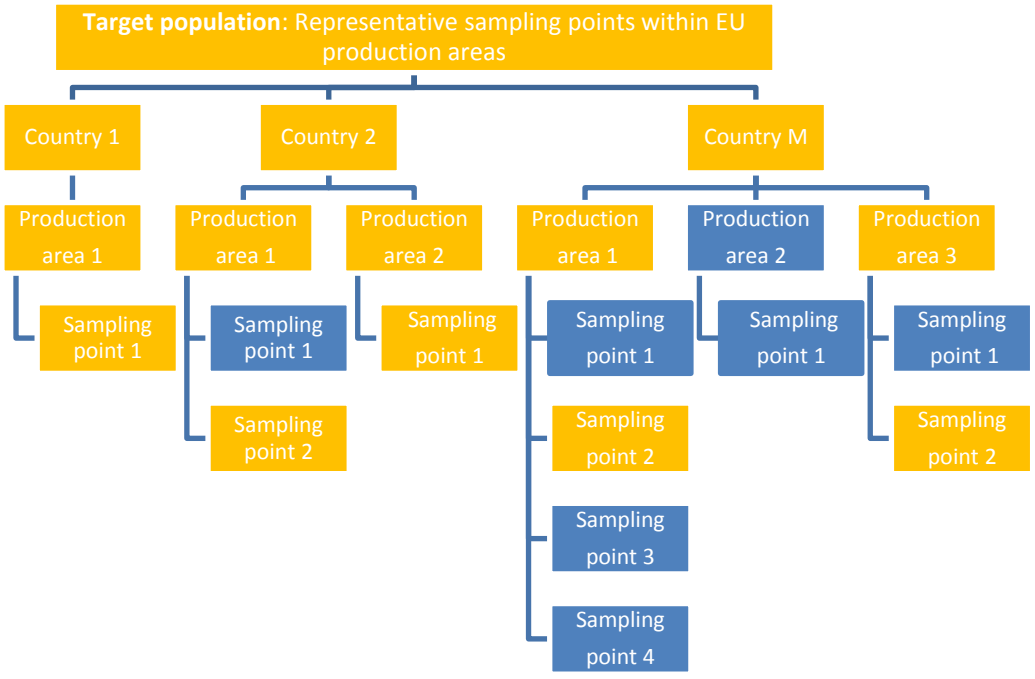

Figure 2: Schematic of the hierarchy within the population for production areas

Every country producing oysters is selected, the countries are considered to be strata and a proportion of production areas are randomly selected within the country, in each production area a single representative sampling point (representing the highest level of *E. coli* contamination) is selected to obtain samples of oysters (yellow indicates inclusion in the survey)

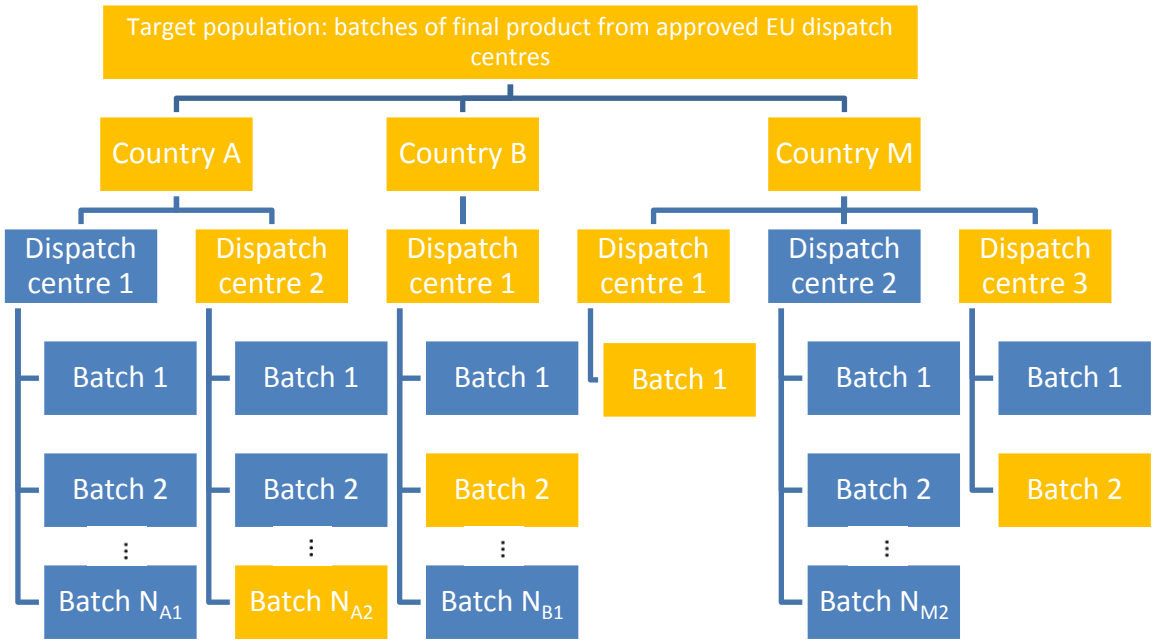

Figure 3: Schematic of the hierarchy within the population for dispatch centres for a two monthly period

Every country dispatching oysters is selected, the countries are considered to be strata and a proportion of dispatch centres are randomly selected within the country, each dispatch centre is considered to be a cluster and a batch of oysters within the dispatch centre is selected to obtain a sample of oysters within a two monthly period (yellow indicates inclusion in the survey)

2.1.3. Sample size

Simple random sample scheme

Sample size calculation formulas presented in this section are based on estimation of the parameter of interest (in this case, the prevalence at EU level of NoV-contaminated oyster active production areas and of NoV-contaminated batches in dispatch centres). The rationale behind the calculation is to first fix the desired margin of error (d), and as d is a function of sample size (n), then by fixing all other parameters, the required sample size can be obtained (Equation 1).

When estimation of the prevalence of NoV in the targeted population is of interest, it is important to achieve the highest precision practically possible. Sample size calculations, considering simple random sampling schemes (SRS), are used to obtain a desired level of precision. The sample size n can be obtained as:

$$n = \frac{z^2\, s^2}{d^2} \qquad (1)$$

where d is the margin of error, defined as the error which the risk manager is willing to accept in estimating the prevalence of NoV-contaminated production areas or batches and z is the normal quantile of α (type I error), the risk that the true margin of error exceeds the acceptable margin of error, (Bartlett, et.al, 2001). s^2 represents the expected variability for SRS, which in this case could be estimated as $s^2 = \bar{\pi} \cdot (1 - \bar{\pi})$, where $\bar{\pi}$ is the expected prevalence.

For the purposes of the NoV baseline survey, α (type I error) is 0.05, the margin of error (d) considered is 0.05 and $s^2 = 0.25$.

Normality assumptions are made implying that sample sizes obtained are based on the consideration of an infinite population. This assumption can be relaxed when dealing with finite populations; the final sample size for a finite population can be adjusted using Equation 2:

$$n^* = \frac{N \cdot n}{N + n - 1} \qquad (2)$$

For the purposes of the NoV baseline survey in both production areas and dispatch centres, the oyster producing countries are considered as strata.

The target population is composed of N units (422 production areas and batches processed in 2,325 dispatch centres during the year of survey) which are divided into 13 strata (Ireland, Greece, Denmark, Sweden, France, Spain, the United Kingdom, the Netherlands, Croatia, Germany, Portugal, Norway and Italy), each of size N_h (Table 1, presents the number of production areas and dispatch centres per country, that are used to construct the weights), $h = 1, \ldots, 13$. Let 'weights' $W_h = \frac{N_h}{N}$ denote the population proportion of the strata. Simple random samples are drawn separately within each stratum.

For the estimation of the population prevalence $\overline{\pi}$, the stratified estimator is given as

$$\overline{\pi}_{st} = W_1\overline{\pi}_1 + W_2\overline{\pi}_2 + \cdots + W_{13}\overline{\pi}_{13} = \sum_{h=1}^{13} W_h\,\overline{\pi}_h\,,$$

with $\overline{\pi}_h$ the stratum sample prevalence. The variance of this estimator, ignoring the finite population correction factor, is expressed as

$$Var(\overline{\pi}_{st}) = \sum_{h=1}^{H} W_h^2 \frac{S_h^2}{n_h} = \sum_{h=1}^{H} W_h^2 \frac{\overline{\pi}_h \cdot (1 - \overline{\pi}_h)}{n_h}, \quad (3)$$

Under proportional allocation ($n_h = \frac{N_h}{N} \cdot n$), similar procedure as the one used to obtain Equation (1) can be applied using Equation (3) resulting in the formula to calculate the sample size for stratified sampling schemes given below

$$n = \frac{z^2}{d^2} \sum_{h} \frac{N_h}{N} S_h^2. \quad (4)$$

Considering finite population correction, the equation to calculate n^* (Equation (2)) is used here with n replaced by Equation (4).

A stratified sampling scheme uses both the proportions provided in Table 1 and previously reported prevalence (if available) to calculate the weights required for the sample size calculation. However, in order to ensure that the proposed sample size accounts for maximum variability, the value used for the expected prevalence for each of the countries is fixed at 0.5.

Under these assumptions, the total sample size for the production areas and batches at dispatch centres in the EU considering the information available and Equation (4), which reduces to equation (1) for an infinite population is:

$$n = \frac{1.96^2}{0.05^2} \times 0.25 = 384.16 \approx 385$$

The number of batches of oysters produced in the EU is large and can be considered to be infinite (Appendix A), however, in May 2015, only 422 production areas were active within Europe. Therefore, production areas can be considered finite. Over a year, there will be changes to the oyster population with repopulation and depopulation occurring. Therefore, the number of production areas is multiplied by a factor for monthly variation (12), resulting in a finite population size of 5,064. The resulting sample size is:

$$n^* = \frac{5064 \cdot 385}{5064 + 385 - 1} = 357.13 \approx 358$$

Using a simple random sampling scheme the total number of samples in the EU to be taken to achieve the precision specified above and adjusted for proportional allocation is 358 for production areas and 385 batches from dispatch centres.

Multistage sample scheme

Simple random sampling ignores some of the complexities inherent in natural systems. The survey design requires a balance between a spatially and temporally representative sampling scheme, the total volume of samples, which can be effectively collected, processed and analysed, and the overall cost of the survey. In the sampling schemes presented above, the sampling units are assumed to be independent, however, this is often not the case especially if multiple samples are taken from a cluster (e.g. production areas and dispatch centres).

The Centre for Environment, Fisheries & Aquaculture Science (CEFAS) survey in 2009/2011 in the UK in oyster production areas indicated that both the prevalence and the level of NoV varied markedly between seasons (Figure 4). Strong winter seasonality was observed. Ninety per cent of the samples taken between October and March were positive compared with 62.4% of samples taken between April and September. Highest levels were detected between December and March (CEFAS, 2011). The EFSA BIOHAZ Panel (2012) also reported strong winter seasonality for NoV contamination in oysters when comparing surveillance data from Ireland, the UK and France. In order to account for temporal variation, multiple samples would need to be taken from the same production area and they may be correlated.

Where sampling units are not independent, the sample size can be obtained by adjusting the variance with the design effect ($[1+\rho(b-1)]$). It is important to highlight that under a cluster sampling design, clusters in the population to be sampled are randomly selected and all elements within a cluster are included. This sampling scheme can be used when the cluster sizes are small. For large clusters, multistage sampling designs are proposed instead. Multistage sampling designs use design effects to ensure that the correlation between sampling units from the same clusters are accounted for when calculating sample size. For multistage designs, sample size can be obtained as:

$$n = [1+\rho(b-1)] \times \frac{z^2 s^2}{d^2} \quad (5)$$

where ρ reflects the correlation between units within same cluster and b reflects the number of elements within a cluster that will be sampled. The remainder of the parameters remain as defined in the simple random sample scheme (Equation (1)).

In order to estimate potential correlation among samples from different months within a production area, data collected in the UK was used (CEFAS, 2011). The resulting model, accounting for seasonality, contains linear and quadratic time effect, as a fixed effect, and a production area as a specific random effect. The results obtained for the fitted model are presented in Figure 4. The variability associated with the random effect (1.54, C.I.: 0.91–1.71) estimated from the final model could be used to estimate the correlation (see p. 27 from EFSA, 2013a) among measurements taken within the production areas to calculate the sample size. The obtained correlation is 0.28 (C.I.: 0.18–0.30), using Equation (5), the design effect, considering sampling bimonthly in each production area selected ($b = 6$, would be 2.4 (C.I.: 1.9–2.5).

Similarly, for dispatch centres the sampling unit is batches, these could be correlated due to similar conditions at the dispatch centre. The sample size calculation needs to account for correlation. Ideally ρ should be known prior to starting the survey, this quantity could be obtained either from pilot studies (as for the case of production areas, in which the CEFAS study was used as a pilot study to estimate the correlation) or based on expert opinion.

Information regarding NoV presence in the French market (Schaeffer et. al., 2013) was used to obtain a proxy for the correlation between batches in a dispatch centre. The results obtained indicate that correlation among samples from the same establishment is likely to be very low (ranging from 0 to 10^6). It should be noted that information on NoV presence in the French market was limited and the samples were taken largely from retailers rather than dispatch centres. Therefore, since no suitable pilot study is available, a multistage sampling scheme was used considering a similar correlation as the one found in production areas (0.30, upper bound), to be conservative.

In order to keep the sampling process manageable and considering that the NoV levels are known to fluctuate across the year, the total number of batches to be sampled and the total number of samples to be taken from a production areas must be evenly distributed along the year. Therefore,

sampling with a bimonthly frequency (once sample every 2 months) is proposed for both production areas and dispatch centres.

The final sample sizes can be calculated using equation (5), considering the cluster size (b) to be 6 (bimonthly visits by inspectors) and the correlation ($\boldsymbol{\rho}$) to be 0.30, upper bound estimate based on the CEFAS (2011) data from production areas. The resulting design effect will be 2.5, which should be further multiplied by the number of production areas or batches to be sampled when considering the simple random sampling scheme (358/385) producing the total number of samples to be taken for production areas and batches from dispatch centres.

For the sample allocation per MS, there is a requirement to consider the possibility that during the survey there may be a failure to take or analyse a sample. For instance, adverse weather conditions, periods with no production in a dispatch centre, problems with sample transport and storage or an invalid analytical result. For this reason, it is necessary to inflate the sample size to ensure the required precision is achieved. For production areas where there should be oysters growing all year round, an inflation of 10% is applied. For dispatch centres, since consumption of oyster is seasonal, there may be months when oysters are not being packed and no sample can be taken, and for this reason, an inflation of 20% is applied.

All calculations were rounded up in order to ensure the level of precision required. Table 2 indicates the number of locations to be visited and the number of samples to be taken annually per country.

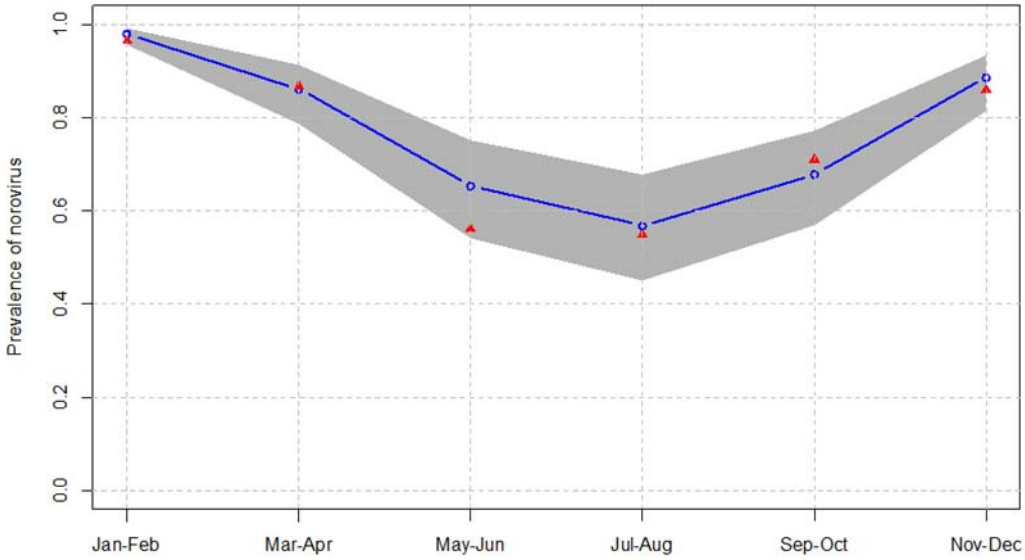

Figure 4: Final fitted model (solid line) together with confidence band (grey shadow) for the fitted model and observed proportions bimonthly (red triangle) from CEFAS (2011).

Table 2: Number of samples to be taken **annually** per country for production areas and dispatch centres (Considering bimonthly samples (once every 2 months) and the inflation for missing data)

	Production area survey		Approved dispatch centre survey	
Country	**Locations**	**Total Samples**	**Locations**	**Total Batches**
Croatia	2	12	1	6
Denmark	3	18	1	6
France	74	444	167	1002
Germany	1	6	1	6
Greece	2	12	1	6
Ireland	22	132	1	6
Italy	7	42	9	54
Netherlands	5	30	3	18
Norway	2	12	0	0
Portugal	5	30	2	12
Spain	14	84	6	36
Sweden	3	18	1	6
United Kingdom	31	186	4	24
Total	**171**	**1026**	**197**	**1182**

2.1.4. Timing of the survey

The result of any baseline survey presents a snapshot of the statistic of interest in the target population for the period when the survey was performed. Surveillance data from many countries indicates that there is annual variation in the number of NoV cases with peaks observed in years when pandemic strains emerge (laboratory reports confirming NoV from England and Wales,[6] notifications and outbreaks data from Ireland,[7] surveillance of acute diarrhoea in France[8] and hospital data admissions from the USA (Hall et al, 2013)). **In order to reduce the probability of surveying an atypical year, it is proposed to repeat the survey for a second year.** Adjustments to the survey in the second year could be made, with agreement from the participating countries, to resolve issues identified in the first survey.

The survey is to take place **simultaneously** at two points in the chain (production areas and dispatch centres) for a period of **2 years**, with samples collected on a **bimonthly** basis (one sample every 2 months). The first samples are to be taken within the period **1 November–31 December 2016**. Since NoV prevalence is subject to temporal variability and the objective of the survey is to estimate the European prevalence, **it is essential that all countries start the survey at the same time**.

2.1.5. Sampling Plan

The CA for participating countries must nominate a national project manager for the baseline survey. The project manager is responsible for

- preparation of a detailed survey plan;
- the designation of one or more laboratories for NoV analysis in consultation with the CA and provide their contact details plus their field of analysis to the EURL;
- the nomination of one or more data providers;

[6] https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/492125/Norovirus_update_2015_week_53.pdf
[7] http://ndsc.newsweaver.ie/epiinsight/epq9pqq0uzsqldxs0g4hal?a=1&p=31757905&t=17517774
[8] https://websenti.u707.jussieu.fr/sentiweb/?page=serie

- development of/modification of existing sampling forms to be used by samplers;
- coordination of the assignment of unique reference numbers for samples;
- ensuring that the required numbers of samples are taken;
- ensuring the survey results have been reported to EFSA.

Prior to commencing the survey, the project manager should draft a detailed survey plan for their country. To assist in the preparation of the survey plan, EFSA will organise a workshop to which all participating countries will be invited. The workshop will focus on finalising country level sampling plans and training in the reporting of the survey results.

In preparation for the workshop, EFSA will send out an excel file to each participating country which combines the information sent on classified production areas and approved dispatch centres to the EURL by May 2015 with the information on monthly batch production requested by EFSA in Nov 2015. These files are to be checked and updated prior to attending the workshop to ensure that the locations in the file match the target populations defined in Section 2.1.1. During the workshop, the files will be imported into a web-based tool to assist in the random selection of locations for inclusion in the survey according the survey design described in Section 2.1.3.

Coordination of the selection of sampling locations is important to ensure that the samples taken are representative for European production areas and batches of final product and therefore ensuring that the European prevalence of NoV-contamination in production areas and batches of final product can be estimated with sufficient precision.

The workshop will also provide the opportunity to discuss any operational issues and ensure that survey is a harmonised process in all participating countries.

2.2. Sample collection

2.2.1. Type and details of sample

The CA considering the availability of samplers and oysters should plan the bimonthly (one sample every 2 months) sampling at the selected locations (dispatch centres and production areas). As far as possible, the locations should be visited on different days in the week and month over the period of the survey. However, consideration should also be given to the requirement for initial processing of the sample by the laboratory to be performed within 72 h of taking the sample, and for samples to arrive at the laboratory during the working week (from Monday to Friday). For example, sampling on Friday should be avoided without prior agreement with the laboratory. Additionally the locations within a geographical region visited at the same time should be varied.

Where more than one oyster species (*O. edulis, C. gigas*, *C. angulata)* is present at the sample site a sample of only one oyster species is required for the survey.

A sample of **15 live oysters** should be taken and dispatched to the designated laboratory for pooled analysis for NoV.

During sampling, precautions should be taken in order to avoid any activity that could affect the levels of viral contamination or result in a sample that is unsuitable for laboratory analysis.

The oysters are to be placed in an intact food grade plastic bag or box (single use), securely packaged, and dispatched to the laboratory with a completed sampling form signed by the sampler and identified by a unique reference number (see Section 2.2.2).

Representative sampling points within harvesting areas

The production areas can be either natural beds or sites used for the cultivation of bivalve molluscs. In some production areas, there may be more than one commercial bivalve mollusc species present. In principle, it is necessary to discriminate between cultivated and wild molluscs, and to take into account the habitat where they grow (intertidal zone, sub tidal zone, benthic, water column, water surface) when selecting representative points.

Regulation 854/2004 highlights the importance of choosing the sampling point/s in production areas based on a survey of faecal pollution inputs, although such information may not necessarily pre-exist for all areas particularly those classified before the applicability of this regulation. It has been

proposed that this information can form the initial basis for assessing the likely extent of NoV contamination of oysters in a harvesting area (Pommepuy and Le Guyader, 2008). EU guidance (EC 2012) provides the possibility to identify one mollusc species as representative (indicator species) of the other ones present in the production area.

For this baseline NoV survey, it is assumed that the oyster species (*O. edulis, C. gigas* and *C. angulata)* are similar in terms of filtration and there is a homogenous distribution of faecal contamination in the proximity of the pollution source between benthic zone and the water column.

When obtaining a sample of oysters for the baseline NoV survey from a production area the following scenarios could occur:

- One or more oyster species are present in the production area and no other molluscs are harvested for human consumption; the representative sampling point used for monitoring activities under Regulation 854/2004 can be used to obtain the sample of oysters.
- More than one species is present in the production area and the oysters are the indicator species; the representative sampling point used for monitoring activities under Regulation 854/2004 can be used to obtain the sample of oysters.
- More than one species is present in the production area but oysters are not the indicator species.
  - If oysters are located in adequate proximity (as described in Section 3.5 of CEFAS, 2014) to the representative sampling point then the representative sampling point used for monitoring activities under Regulation 854/2004 can be used to obtain the sample of oysters.
  - If the oysters are not located in adequate proximity to the representative sampling point is it necessary to identify and define a new sampling point within the area of oyster production that has the highest risk of faecal pollution based on the sanitary survey. The point must be fixed using the criteria reported in the EU guidance (EC 2012) and should be used to obtain the sample of oysters.

Where multiple representative sampling points are present in the same production area, the representative sampling point, where oyster species are present, with the highest levels of *E. coli* contamination based on the routine monitoring performed under Regulation 854/2004 should be used to obtain the sample of oysters.

Oysters can be placed at the sampling point, at the direction of the CA, to facilitate sampling for the purposes of monitoring. It is important that the oysters sampled, have been growing in the selected production area for more than 28 days, in order to be representative of this area and not a previous one.

Samples are to be collected by samplers in parallel to the collection of samples for the microbiological monitoring programme, as specified by Regulation 854/2004. An additional sample of 15 live oysters is required for the baseline survey.

Approved dispatch centres

Oyster samples should be collected by samplers according to official controls procedures. The sample should be taken from one batch of live oysters present on the premises at the time of visit. Fifteen oysters should be selected from the boxes on the packing line (representative of the sizes and grades of animals in the batch). The sample should not contain a mix of oyster species as this would not conform to the definition of a batch. It is acknowledged that 15 oysters may not be sufficient to detect NoV contamination in a batch, if the proportion of oysters contaminated is less than 20% (assuming test sensitivity greater than 90%). However, for countries with a large number of dispatch centres the number of oysters that can be processed by the laboratory daily is limited. In addition, there is the issue of the commercial value of oysters at this point in the food chain.

The documents should be checked according to the record keeping requirements of Annex III, Section VII, Chapter I, and points 3 to 7 of Regulation (EC) No 853/2004 and special attention must be paid to the traceability records of the selected batch, from the Identification Mark back to the registration documents recording relaying or depuration records (if applicable). If the production area/s for the batch cannot be identified then the sample should not be taken.

2.2.2. Sample information

For the purposes of the survey, each sample should have a **unique reference number**. This number should be used to ensure that the information collected by the sampler could be linked with all of the analytical results generated in the laboratory. This reference number is required for reporting data to EFSA and will be used for the subsequent data analysis. This number will also be used to calculate the number of samples performed per country upon completion of the survey. Depending on the data management systems in place in the country, this can be printed on the sample form or alternatively an identifier can be generated upon receipt of the sample at the laboratory.

The principles for stipulating data to be recorded for this survey are as follows:

- the information will be used for estimating the prevalence, or analysing the risk factors that might contribute to that prevalence;
- the information should be readily obtainable by an official inspector.

Production Area: The types of data that should be available regarding oysters taken from production areas are described in general in traceability requirements of Regulation (EC) No 178/2002[9] and its implementing regulation 931/2011.[10] Oysters are also subject to the more specific requirements for all fishery and aquaculture products in the Fishery Control regulation 1224/200 [11] and its implementing regulation 404/2011. [12] However, the key piece of legislation setting out the information that should be readily available for LBM coming from production areas is the Shellfish Registration Document required by Regulation (EC) No 853/2004. Of particular note is the requirement for the classification status (based on *E. coli* monitoring results) of the production area to be indicated.

Dispatch Centres: Batches of oysters from dispatch centres may comprise the products of several different production areas. Such food batches are subject to the general traceability and specific fishery control legislation in addition to Regulation (EC) No 853/2004. The general applicability of the Regulation 1169/2011[13] on Food Information to Consumers is also relevant at this stage. However, in the present context, the key piece of legislation is Regulation 1379/2013[14], which sets out the mandatory information that should be present on fishery and agriculture products when labelled for consumer sale or supply to mass caterers. Even if batches going from dispatch centres have not been labelled for such markets, they should be accompanied by such information in order to ensure accuracy of subsequent labelling. Labelling requirements are summarised in the EU Commission guide.[15] Some provisions directly relevant to this programme include the mandatory requirement to provide catch area and catch method for wild-caught fish, and the provision of origin information for farmed fish, which in the case of farmed shellfish is the country in which it underwent a final rearing or cultivation stage of at least 6 months.

A sample form to record the information listed below should be developed (examples of the sample forms are available in Appendix C.

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9 Regulation (EC) No 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.

10 Commission Implementing Regulation (EU) No 931/2011 on the traceability requirements set by Regulation (EC) No 178/2002 of the European Parliament and of the Council for food of animal origin.

11 Council Regulation (EC) No 1224/2009 establishing a Community control system for ensuring compliance with the rules of the common fisheries policy, amending Regulations (EC) No 847/96, (EC) No 2371/2002, (EC) No 811/2004, (EC) No 768/2005, (EC) No 2115/2005, (EC) No 2166/2005, (EC) No 388/2006, (EC) No 509/2007, (EC) No 676/2007, (EC) No 1098/2007, (EC) No 1300/2008, (EC) No 1342/2008 and repealing Regulations (EEC) No 2847/93, (EC) No 1627/94 and (EC) No 1966/2006.

12 Commission Implementing Regulation (EU) No 404/2011 laying down detailed rules for the implementation of Council Regulation (EC) No 1224/2009 establishing a Community control system for ensuring compliance with the rules of the Common Fisheries Policy.

13 Regulation (EU) No 1169/2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

14 Regulation (EU) No 1379/2013 on the common organisation of the markets in fishery and aquaculture products, amending Council Regulations (EC) No 1184/2006 and (EC) No 1224/2009 and repealing Council Regulation (EC) No 104/2000.

15 http://trade.ec.europa.eu/doclib/docs/2014/december/tradoc_152941.pdf

Production Area Samples

- **sample reference number;**
- country:
- production Area Code and/or Production Area name (Appendix D);
- classification at time of sampling:
  - A, B or C (at time of sampling);
  - fully classified, Seasonal or Preliminary (optional);
- sampling Date and Time
- oyster species (*O. edulis, C. gigas or C. angulata*)
- sampling location (Longitude and latitude of representative sampling point (WGS84 format)):
  - intertidal – zone of shore between the high-water mark and the low-water mark;
  - or Inshore < 5km from shore;
  - or Offshore >= 5km from shore;
- production information:
  - farmed or Wild;
  - production system:
    - raised trestle – bagged oysters grown on a raised structure (table, rack or trestle) installed on the substrate, on the foreshore;
    - or suspended from sea-surface – oysters are permanently immersed and suspended from tables or buoys using nets, caskets or ropes;
    - or bottom grown – oysters produced from existing oyster beds;
- remarks, any unusual conditions at time of sampling or deviations from sampling plan;
- sampler name (this is only required within the country in case of missing information and will not be reported to EFSA).

Dispatch Centre Samples

- **sample reference number;**
- country;
- dispatch centre approval number (Appendix D);
- sampling date and time;
- oyster species (*O. edulis, C. gigas or C. angulata*);
- overall batch weight (kgs);
- overall batch origin (production area/s or catch area/s most representative of origin in terms of quantity (Articles 35 (3) of Regulation (EC) 1379/2013);
- number of production areas that contributed to sampled batch.

  For each contributing production area 'lot' of fish:
  - production Area Code and/or Production Area name (Appendix D), in case of relaying this should not be the relaying area code but the production area code prior to relaying;
  - classification of production area as indicated in traceability documents:
    - A, B or C;
  - date of harvesting;
  - indicate if any of the following activities or treatments have been applied to the oysters in the batch and the **duration**:

        - conditioning;
        - relaying;
        - purification:
            - If purified describe conditions:
                - Ambient, actively heated, actively cooled or Unknown
                - Temperature (°C)
    - indicate if the oysters are wild or farmed:
        - If farmed, origin as defined by Regulation 1379/2013 Article 38 1(c). Country in which the oysters underwent a final rearing or cultivation of at least 6 months
        - If wild, at least FAO area code or more accurate indicator of origin, e.g. country coastal waters, and whether the oysters were hand-picked, raked or dredged
- Record if the batch contains oysters that have already been assigned an Identification Mark in cases where the dispatch centre is performing mixing of splitting activities
- Remarks, any unusual conditions at time of sampling or deviations from sampling plan
- Sampler name (this is only required within the country in case of missing information and will not be reported to EFSA)

2.2.3. Sample transport

Sample transport from both production areas and dispatch centres must be carried out in accordance with the requirements laid out in Section 4 of CEFAS (2014) with the exception that the maximum time permitted between sample collection and the initiation of sample processing is 72 h. For this reason, the time of sampling must be recorded on the sample form. When taking samples it is important to consider that samples should arrive at the laboratory during the working week.

2.3. Laboratory analysis

2.3.1. Participating laboratories

The baseline survey is envisaged as a formal activity of CA falling under their Official Control responsibilities. Therefore, the working group has reviewed the usual requirements for Official Control sampling and analysis as set out in Regulation 882/2004[16] for applicability to this baseline survey.

Designation of laboratories: To ensure laboratories are under the responsibility of the CA, EU Regulation 882/2004 requires them to be formally designated by the CA. Therefore, the CA in each country must designate the laboratories undertaking NoV analysis for this survey. The designated laboratories can perform one or more of the following steps described in the EURL method specification (Appendix B); sample preparation, virus extraction, RNA extraction, PCR detection, PCR quantification or sample archiving.

The following conditions apply to designated laboratories:

- a protocol must be prepared for the analysis the laboratory will perform in the survey. If more than one laboratory is involved in producing an analytical result, the protocol(s) must describe the parts undertaken by each laboratory, the conditions for transport of processed samples between laboratories, and any necessary breakpoints, etc. Protocol(s) must be in conformity with the method specification in Appendix B and must be approved by the National Reference Laboratory (NRL). Protocol(s) covering the entire analytical procedure must form an annex of the survey plan prepared by the project manager;
- the laboratory does not need to be accredited for ISO/DIS 15216-1; however, **laboratories undertaking quantification must be able to report an LOQ for the NoV PCR**. For laboratories requiring assistance, approaches to establishing an LOQ will be covered as part of the optional EURL training (detailed below);

[16] Regulation (EC) No 882/2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules.

- the laboratories performing NoV quantification must participate in the EURL proficiency test. Laboratories undertaking other aspects of the method should participate in proficiency testing organised by their NRL.

Supervision by the NRL: To ensure harmonised analysis at the EU level, Regulation 882/2004 sets out the responsibilities of NRLs for supervision of Official Control laboratories. It is proposed to adopt the same approach for this survey – designated laboratories must be supervised by the NRL of that MS. This requirement is not necessary if the designated laboratory is the NRL. Following designation, the EURL will contact all laboratories designated for NoV analysis, and supervising NRLs to further discuss training and analysis. For laboratories not performing PCR quantification, as part of the supervisory role of the NRL, the NRL may organise training and local proficiency tests to ensure the necessary standards can be achieved by these laboratories.

Training: NoV analysis is complex and proficiency testing clearly demonstrates the potential for significant deviation of results between laboratories following analysis of the same distributed sample. It is considered essential for this harmonised survey that a high level of confidence can be ascribed to the comparability between laboratories for results of NoV analysis (both qualitative and quantitative). EURL proficiency testing clearly demonstrates that robust confidence in analytical comparability is best achieved through the adoption of harmonised analytical methods and by ensuring laboratory competence. To assist the adoption of harmonised procedures, and to reinforce competence, the EURL will offer an EC funded 4-day training course open to at least one technician from all designated laboratories undertaking analysis of NoV for this survey. This training is not mandatory. The NRLs may also organise a similar training for technicians from the designated laboratories within their country.

Competence assessment: Following the above training, and for those laboratories undertaking quantification, the EURL will organise a proficiency test round to assess laboratory competence. Where analysis is split between laboratories, NRLs should also perform proficiency testing to ensure competence in all parts of the method. Laboratories will be given additional support by the NRL and EURL in case of the requirement of a repeat of the proficiency test. To participate in the NoV PCR quantification for the survey, it will be necessary to demonstrate satisfactory performance in proficiency testing.

2.3.2. Analytical method

The analysis of NoV in oysters by all laboratories will be performed according to the method specification developed by the EURL expert working group specifically for this study (Appendix B). The survey requires separate real-time reverse transcription (RT)-PCR analysis for NoV genogroup I (GI) and NoV genogroup II (GII). This method specification sets out the procedures that designated laboratories are required to follow in performing analysis of oysters submitted under this survey. The specification is based on, and is compliant with, ISO/DIS 15216-1; Microbiology of the food chain -- Horizontal method for determination of hepatitis A virus and NoV in food using real-time RT-PCR -- Part 1: Method for quantification (the draft revision to the ISO method including validation data, due for publication in 2016). Accordingly, there are some differences to the current published technical specification ISO/TS 15216-1:2013. The method specification has been compiled and agreed by the EURL technical working group comprised of representatives from the EURL and EFSA, and NRLs of France, Ireland, Germany, Denmark and Italy.

Use of the procedures in compliance with the method specification will be mandatory for all laboratories participating in NoV analysis in the study. The MS NRL will supervise analysis in the designated laboratories and will be responsible for ensuring compliance with the protocol. Upon request, control materials can be provided to the participating laboratories by the EURL.

2.3.3. Storage of samples

All RNA extracts should be stored as described in the method specification (Appendix B). This would allow reanalysis in case of reagent or standard failures or other issues related to the estimation of NoV copy number.

It is proposed to archive all samples collected during this project to facilitate further research (e.g. NoV whole genome sequencing, analysis for presence/absence of hepatitis A virus). Once the analytical result has been confirmed the digestive glands, supernatant and RNA should be stored

at **-70^{0}C** or below. These samples should be stored at a single location in each country, either the NRL or another laboratory with suitable archiving facilities. The samples should be **stored for a minimum of 2 years** after the survey has been completed. The unique reference number used in the survey must be linked to all of the stored samples so that the survey data can also be used in further research efforts.

Researchers wishing to make use of the baseline survey sample archive must present their research proposals to the Standing Committee on Plants, Animals, Food and Feed (PAFF Committee). They must seek permission for the use of the samples in the archive from the relevant countries holding the samples from the PAFF Committee. These proposals must be made within 1 year from the completion date of the survey, and the research should be performed within the following year. Priority for use of the samples will be given to European research proposals; however, the remaining materials can be used for national research initiatives.

2.4. Reporting

The national project manager should nominate one or more data providers responsible for the submission of the results of the baseline survey to EFSA and the European Commission. The data providers can be staff from the nominated laboratories, the CA or other agencies with a responsibility for microbiological monitoring of LBM; however, where there are multiple data providers the survey project manager should ensure that the reporting is consistent within the country.

The nominated data providers will be provided with instruction manuals and training in the preparation and submission of the data by EFSA. The nominated data providers will be the contact points in case of data validation and data quality queries.

It is essential that sample forms are checked for completion and all the information on the form is entered correctly into the local data management system.

2.4.1. Data model

EFSA Standard Sample Description version 2.0 (EFSA, 2013b) is designed to allow the reporting of laboratory results that are comparable between laboratories and countries. The reporting of the baseline survey results will use this data model as the basis with extensions to support the analysis of the results of the baseline survey (Table 3 and 4). The application of controlled terminology ensures that the reporting of results is harmonised and comparable between countries. The Controlled Terminology column of Table 3 and 4 indicates those variables where a controlled terminology is applied. Where a limited number of terms can be selected, the codes or terms to be used are specified in full and in cases where a longer list of terms can be selected (e.g. country of origin), the catalogue names are listed (underlined). These catalogues are published on the EFSA website.[17]

In the tables below, the variables to be reported for each laboratory result are described. A unique identifier (resID) is required for each row within the dataset. One row represents a single analytical result linked to a description of the sample of oysters. There can be more than one analytical result for each oyster sample; therefore, the unique reference number (sampID) must be the same for all analytical results for that sample of oysters. The quantitative results and results below limit of detection (LOD)/LOQ for both genotype I and genotype II must be reported for all samples taken. If it is not possible to analyse the sample or a valid laboratory result cannot be obtained the reason for this failure should be recorded.

[17] http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3424.pdf

Table 3: Production areas: Description of variables to be reported for the survey

Element Name	Data Type	M[(a)]	Controlled terminology	Description
progId	xs:string (100)	Y	EU baseline survey of norovirus in oysters	Name of survey
progLegalRef	xs:string (5)	Y	LEGREF	Reference to published Commission Decision concerning financial contribution
sampStrategy	xs:string (5)	Y	ST10A = Objective sampling (SRS)	Description of the sampling methodology
progType	xs:string (5)	Y	K027A = Survey - EU baseline survey	Type of programme for which the samples have been collected
sampPoint	xs:string (5)	Y	E150A = Production areas	Specify that the sample came from a production area
sampId	xs:string (100)	Y		The **unique reference number** for the sample, this must be maintained when reporting all laboratory results linked to the sample See Section 2.2.2
sampUnitType	xs:string (5)	Y	G198A = holding/production area	Description of the sampling unit
sampUnitSize	xs:double	Y		Mean animal weight in sample (entire animal including shell) sample weight/number of oysters
sampUnitSizeUnit	xs:string (5)	Y	G167A = grams	Units for the value reported in sampSizeUnit
sampHoldingId	xs:string (250)	Y	Production area code list in (Appendix D)	Site identifier for the production area the sampled oysters came from
areaStatus	xs:string (5)	Y	A / B / C	Classification of the production area at time of sampling
classType	xs:string (250)		Fully classified / Seasonal / Preliminary	Type of classification applied for the areaStatus reported above
areaType	xs:string (250)	Y	Intertidal / Inshore / Offshore / Unknown	Type of oyster production area
production	xs:string (250)	Y	Wild / Farmed / Unknown	Indicate if the oysters are wild or farmed
productionsystem	xs:string (250)	Y	Raised Trestle / Suspended from sea-surface / Bottom-grown / Unknown	Description of the oyster production system
sampCountry	xs:string (2)	Y	COUNTRY	Country where the sample was taken for laboratory testing (ISO 3166-1-alpha-2)
longitude	xs:string (20)			Longitude of the location of the representative sampling point where the sample was taken in WGS84 format
latitude	xs:string (20)			Latitude of the location of the representative sampling point where the sample was taken in WGS84 format
sampY	xs:integer (4)	Y		Year of sample
sampM	xs:integer (2)	Y		Month of sample
sampD	xs:integer (2)	Y		Day of sample
sampT	xs:integer (2)			Hour of sampling in 24-h format

Element Name	Data Type	M[(a)]	Controlled terminology	Description
sampInfo	xs:string (250)			Report any remarks recorded at the time of sampling
arrivalD	xs:integer (2)			Day of arrival in the lab
arrivalT	xs:integer (2)			Hour of arrival in the laboratory in 24-h format
arrivalTemp	xs:double	Y		Sample temperature at time of arrival in the lab in Centigrade
sampMatType	xs:string (5)	Y	S019A = Food sample	Type of sample taken
sampMatCode	xs:string (400)	Y	A055F = *Ostrea edulis* A055J = *Crassostrea gigas* A02HK = *Crassostrea angulata*	Species of oyster sampled
sampMatText	xs:string (250)			Optional description of the characteristics of the sample taken using free text
origFishAreaCode	xs:string (10)	Y	FAREA	Fisheries or aquaculture area specifying the origin of the sample (FAO Fisheries areas) at the level of FAO Sub Area
sampAnId	xs:string (100)			Identification code of the analysed sample, by default the same as the sampId. Used when the sample is split into analysis portions for the same analytical measurement, in this case sampID plus incremental number
analysisY	xs:integer (4)	Y		Year when the analysis was completed
analysisM	xs:integer (2)	Y		Month when the analysis was completed
analysisD	xs:integer (2)	Y		Day when the analysis was completed
labId	xs:string (50)	Y		Identification code of the laboratory to be contacted in case of questions about the result (National laboratory code if available). This code should be nationally unique and consistent through all data domain transmissions.
NRL	xs:string (1)	Y	Y / N	Is the laboratory the national reference laboratory
labCountry	xs:string (2)	Y	COUNTRY	Country where the laboratory is located (ISO 3166-1-alpha-2).
paramCode	xs:string (400)	Y	RF-00003060-PAR = Norovirus GI RF-00003061-PAR = Norovirus GII	Analysis for which microorganism. For each sample the results for both norovirus GI and GII should be reported as separate rows
paramText	xs:string (250)			Description of the parameter/ analyte using free text
anMethRefCode	xs:string (5)	Y	R023A = CEN ISO TS 15216-1:2013	Code to identify the analytical method used
anMethText	xs:string (250)			Description of the method if any deviation from the validated method occurred
resId	xs:string (100)	Y		Identification code of an analytical result (a row of the data table) in the transmitted file. The result identification code must be

Element Name	Data Type	M[(a)]	Controlled terminology	Description
				maintained at organisation level and it will be used in further updated/deletion operation from the senders.
accredProc	xs:string (5)		V001A = Accredited according to ISO/IEC17025 V005A = Internally validated V999A = Not validated	The accreditation status of the analytical method
resUnit	xs:string (5)	Y	Detectable virus genome copies per g	Unit of measurement the result value
resLOD	xs:double			Report the limit of detection
resLOQ	xs:double	Y		Report the limit of quantification
resVal	xs:double			The quantitative result not censored according to the LOQ; Detectable norovirus genome copies per g
resQualValue	xs:string (3)		POS = positive NEG = not detected	Report the qualitative result of the laboratory analysis
resType	xs:string (3)		VAL =sample is quantifiable LOQ = sample is not quantifiable	For positive results, indicate whether the sample is quantifiable or not quantifiable
resValUncert	xs:double			Indicate uncertainty of measurement log 10 copies/g for QPCR
resValUncertSD	xs:double			Standard deviation for the uncertainty of measurement.
resStatus	xs:string (50)	Y	Valid / Not valid/ Not tested	Each sample collected should be reported Select 'Not Tested' if the sample was rejected for laboratory testing Select 'Not Valid' if no laboratory result could be obtained following retesting
resInfo	xs:string (250)			Where resStatus is not valid report the reason quantification of NoV could not be achieved

(a): 'Y' indicates this element is mandatory and must be completed

Table 4: Dispatch centres: Description of variables to be reported for the survey

Element Name	Type	M[(a)]	Controlled terminology	Description
progId	xs:string (100)	Y	EU baseline survey of norovirus in oysters	Name of survey
progLegalRef	xs:string (5)	Y	LEGREF	Reference to published Commission Decision concerning financial contribution
sampStrategy	xs:string (5)	Y	ST10A = Objective sampling	Description of the sampling methodology
progType	xs:string (5)	Y	K027A = Survey - EU baseline survey	Type of programme for which the samples have been collected
sampPoint	xs:string (5)	Y	E320A = Approved dispatch centre	Specify that the sample came from a dispatch centre

Element Name	Type	M[(a)]	Controlled terminology	Description
sampId	xs:string (100)	Y		The **unique reference number** for the sample, this must be maintained when reporting all laboratory results linked to the sample See Section 2.2.2
sampUnitType	xs:string (5)	Y	G204A = batch	Description of the sampling unit
sampUnitSize	xs:double	Y		Overall batch weight
sampUnitSizeUnit	xs:string (5)	Y	G167A = kilograms	Units for the value reported in sampSizeUnit
sampPlantId	xs:string (250)	Y	Dispatch centre code list in (Appendix D)	Dispatch Centre Approval Number the sampled consignment came from
batchId	xs:string (250)	Y		Report the unique identifier of the batch from the internal traceability system
sampHoldingId	xs:string (250)	Y	Production area code list in (Appendix D)	Site identifier for the production areas the sampled oysters came from. Where a batch is composed of oysters from more than one production area the codes should be listed separated by a '$'
nProductionAreas	xs:integer (4)	Y		Report the number of production areas the oysters in batch came from
areaStatus	xs:string (5)		A / B / C	Classification of the production areas that were the source of the batch Where a batch is composed of oysters from different production areas, the area statuses should be listed separated by a '$'
production	xs:string (250)	Y	Wild / Farmed / Unknown	Indicate if the oysters are wild or farmed
repacked	xs:string (1)	Y	Y / N / U	Indicate if any oysters within the batch have already been assigned with an Identification Mark – i.e. the dispatch centre is performing mixing or splitting activities
sampCountry	xs:string (2)	Y	COUNTRY	Country where the sample was taken for laboratory testing (ISO 3166-1-alpha-2).
sampY	xs:integer (4)	Y		Year of sample
sampM	xs:integer (2)	Y		Month of sample
sampD	xs:integer (2)	Y		Day of sample
sampT	xs:integer (2)			Hour of sampling in 24-h format
sampInfo	xs:string (250)			Report any remarks recorded at the time of sampling including any issues related to the traceability documentation
arrivalD	xs:integer (2)			Day of arrival in the lab
arrivalT	xs:integer (2)			Hour of arrival in the laboratory in 24-h format
arrivalTemp	xs:double	Y		Sample temperature at time of arrival in the lab in Centigrade

Element Name	Type	M[(a)]	Controlled terminology	Description
sampMatType	xs:string (5)	Y	S019A = Food sample	Type of sample taken
sampMatCode	xs:string (400)	Y	A055F = *Ostrea edulis* A055J = *Crassostrea gigas* A02HK = *Crassostrea angulata*	Species of oyster sampled
sampMatText	xs:string (250)			Optional description of the characteristics of the sample taken using free text.
durCondition	double	Y		Report the duration of conditioning applied to oysters in the batch in **hours** 0 indicates the batch has not be subjected to conditioning **-9999** indicates it is not known if the batch has been subjected to conditioning
durRelaying	double	Y		Report the duration of relaying applied to oysters in the batch in **days** **0** indicates the batch has not be subjected to relaying **-9999** indicates it is not known if the batch has been subjected to relaying
durPurification	double	Y		Report the duration of purification applied to oysters in the batch in **hours** **0** indicates the batch has not be subjected to purification **-9999** indicates it is not known if the batch has been subjected to purification
condPurification	xs:string (250)		Ambient / Actively heated / Actively cooled / Unknown	Report the thermal status of the water used for purification if purification has occurred
purificationTemp	xs:double			Temperature of water in purification tank in Centigrade if purification occurred
origCountry	xs:string (2)		COUNTRY	Overall Batch Origin (production area/s or catch area/s most representative of origin in terms of quantity (Articles 35 (3) of Regulation (EC) 1379/2013)
origFishAreaCode	xs:string (10)		FAREA	For wild oysters, report the origin of the sample using at the level of FAO sub area
origFishAreaText	xs:string (250)			Name of the fishing area specified on the label. Where a batch is composed of oysters from more than one area the names should be listed separated by a '$'
fishMethod	xs:string (250)		hand-picked / raked / dredged / unknown	Fishing method used to obtain oyster batch for wild oysters

Element Name	Type	M[(a)]	Controlled terminology	Description
procCountry	xs:string (50)		COUNTRY	If farmed, origin As defined by Regulation 1379/2013 Article 38 1. (c). Country in which the oysters underwent a final rearing or cultivation of at least 6 months. Where a batch is composed of oysters from more than one country the codes should be listed separated by a '$'
prodY	xs:string (4)			Year of harvesting of the oysters included in the batch
prodM	xs:string (10)			Month of harvesting of the oysters included in the batch. If there are different harvesting months (oysters from more than source) the months should be listed separated by a '$'
prodD	xs:string (10)			Day of harvesting of the oysters included in the batch. If there are different harvesting days (oysters from more than source) the days should be listed separated by a '$'
sampAnId	xs:string (100)			Identification code of the analysed sample, by default the same as the sampId. Used when the sample is split into analysis portions for the same analytical measurement, in this case sampID plus incremental number
sampSize	xs:double	Y		
sampSizeUnit	xs:string (5)	Y	G167A = grams	Units for the value reported in sampSize
analysisY	xs:integer (4)	Y		Year when the analysis was completed.
analysisM	xs:integer (2)	Y		Month when the analysis was completed.
analysisD	xs:integer (2)	Y		Day when the analysis was completed.
labId	xs:string (50)	Y		Identification code of the laboratory (National laboratory code if available). This code should be nationally unique and consistent through all data domain transmissions.
NRL	xs:string (1)	Y	Y / N	Is the laboratory the national reference laboratory
labCountry	xs:string (2)	Y	COUNTRY	Country where the laboratory is located (ISO 3166-1-alpha-2).
paramCode	xs:string (400)	Y	RF-00003060-PAR = Norovirus GI RF-00003061-PAR =Norovirus GII	Analysis for which microorganism. For each sample, the results for both norovirus GI and GII should be reported as separate rows.
paramText	xs:string (250)			Description of the parameter/ analyte using free text
anMethRefCode	xs:string (5)	Y	R023A = CEN ISO TS 15216-1:2013	Code to identify the analytical method used

Element Name	Type	M[(a)]	Controlled terminology	Description
anMethText	xs:string (250)			Description of the method if any deviation from the validated method occurred
resId	xs:string (100)	Y		Identification code of an analytical result (a row of the data table) in the transmitted file. The result identification code must be maintained at organisation level and it will be used in further updated/deletion operation from the senders.
accredProc	xs:string (5)		V001A = Accredited according to ISO/IEC17025 V005A = Internally validated V999A = Not validated	The accreditation status of the analytical method
resUnit	xs:string (5)	Y	Detectable virus genome copies per g	Unit of measurement the result value
resLOD	xs:double			Report the limit of detection
resLOQ	xs:double	Y		Report the limit of quantification
resVal	xs:double			The quantitative result not censored according to the LOQ; Detectable norovirus genome copies/g
resQualValue	xs:string (3)		POS = positive NEG = not detected	Report the qualitative result of the laboratory analysis
resType	xs:string (3)		VAL =sample is quantifiable LOQ = sample is not quantifiable	For positive results, indicate whether the sample is quantifiable or not quantifiable
resValUncert	xs:double			Indicate uncertainty of measurement log 10 copies/g for QPCR
resValUncertSD	xs:double			Standard deviation for the uncertainty of measurement
resStatus	xs:string (50)	Y	Valid / Not valid/ Not tested	Each sample collected should be reported Select 'Not Tested' if the sample was rejected for laboratory testing Select 'Not Valid' if no laboratory result could be obtained following retesting
resInfo	xs:string (250)			Where resStatus is not valid report the reason quantification of NoV could not be achieved

(a): 'Y' indicates this element is mandatory and must be completed

2.4.2. Business rules

The submitted data will be checked to ensure that the mandatory elements (Column M in Tables 3 and 4) are completed, the correct data type is used and for compliance with the controlled terminologies. In addition, checks between elements will be made for consistency and plausibility. The checks are listed in Table 5 including a description of the business rule code and message reported in case a record fails the business rule checks. If the submitted data set fails, one or more of the checks the data set is rejected. A report listing the identified errors will be sent to the data provider with advice on required modifications. The data provider must resubmit the data until the data set passes the validation checks and is marked as 'Valid'.

Table 5: Business rules for reporting baseline survey results

Description	Elements	Info Type	Business rule code	Info Message
'Year of analysis' has to be less than or equal to the current year	analysisY$<=$nowY	E	ER1	Analysis year cannot be greater than the current year
The 'area of sampling' reported must be included in the country reported in 'Country of sampling'	sampArea$sampCountry	E	ER4	The sampling area must be within the sampling Country
'Year of sampling' must be less or equal to the current year	sampY$<=$nowY	E	ER5	Sample year cannot be greater than the current year
'Year of sampling' must be less than or equal to 'year of analysis'	sampY$<=$analysisY	E	ER6	Sample year cannot be greater than the analysis year
'Month of sampling' has to be filled in if 'Day of sampling' is filled in	sampM$sampD	E	ER8	Sample month must be completed if sample day is completed
The partial date sampM/sampY must be less or equal to the current partial date M/Y	sampM$sampY$<=$nowM $nowY	E	ER9	The combination of sample month and sample year must be less than or equal to the current month and year
The partial date sampM/sampY must be less or equal to the partial date analysisM/analysisY	sampM$sampY$<=$analysi sM$analysisY	E	ER10	The combination of sample month and sample year must be less than or equal to the analysis month and year
The date sampD/sampM/sampY must be a valid date	sampD$sampM$sampY	E	ER12	The combination of sample day, month and year must be a valid date
The date sampD/sampM/sampY must be less or equal to the current date D/M/Y	sampD$sampM$sampY$<=$nowD$nowM$nowY	E	ER13	The combination of sample day, month and year must be less than or equal to the current date
The date sampD/sampM/sampY must be less or equal to the current date analysisD/analysisM/analysisY	sampD$sampM$sampY$<=$analysisD$analysisM$analysisY	E	ER14	The combination of sample day, month and year must be less than or equal to the analysis day, month and year
Where resType = "VAL" then resVal must be provided	resVal$resType$=$"VAL"	E	ER15	The result value must be completed if the sample is quantifiable
Where resQualValue = "POS" then resType must be provided	resType$resQualValue$=$"POS"	E	ER16	The result type must be completed if the sample is reported as positive
Where resStatus = "Valid" then resQualValue must be provided	resQualValue$resStatus$=$"Valid"	E	ER17	If the laboratory result is valid then the qualitative result must be reported
Where durPurification > 0 then condPurification must be provided	condPurification $ Not Tested $>$0	E	ER18	The purification conditions must be reported when the duration of purification (durPurification) is greater than 0
Where resStatus = "Not Tested" then resInfo must be provided	resInfo$resStatus$=$" Not Tested"	E	ER19	The resInfo must be completed if resStatus is 'Not tested'

Description	Elements	Info Type	Business rule code	Info Message
Where resStatus = "Not Valid" then resInfo must be provided	resInfo$resStatus$=$" Not Valid"	E	ER20	The resInfo must be completed if resStatus is 'Not Valid'
Where production = "Wild" then fishMethod must be provided	fishMethod$production$=$ "Wild"	E	ER21	The fishMethod must be completed if production is 'Wild' for batches from dispatch centres
Where production = "Wild" then origFishAreaText must be provided	origFishAreaText$production$=$"Wild"	E	ER22	The origFishAreaText must be completed if production is 'Wild' for batches from dispatch centres
Where production = "Wild" then origFishAreaCode must be provided	origFishAreaCode$production$=$"Wild"	E	ER23	The origFishAreaCode must be completed if production is 'Wild' for batches from dispatch centres
Where production = "Farmed" then procCountry must be provided	procCountry$production$=$"Farmed"	E	ER24	The country where the oysters underwent final rearing must be reported if production is 'Farmed' for batches from dispatch centres
'Mean weight of oysters' must be less or equal to the 250	sampUnitSize $<=250	E	ER25	The mean weight of the oysters samples cannot be greater than 250 g

2.4.3. Submission to Data Collection Framework

The data should be reported via the EFSA data collection framework (DCF).[18] Data received by the EFSA DCF will go through automated validation processes for data elements, controlled terminology values, and general and specific business rules (described in Section 2.4.2). Messages regarding the status of the transmission will be exchanged with the data provider.

The format for data submission is Extensible markup language (XML).[19] EFSA will provide excel templates to assist in the compilation of the results of the baseline survey and facilitate the export of the data into XML format. The use of the templates is not mandatory; data providers can chose to use their own data management systems to output the results in the required XML format. The XML schema definitions[20] describing the required XML format are available in Appendix D.

The available results of laboratory testing performed for the baseline survey should be reported on a quarterly (every 3 months) basis. Where RNA extracts are stored prior to batch laboratory analysis, the results for all samples within the batch should be reported as soon as the real-time RT-PCR quantification is completed. This will allow early validation of the data to ensure that the problems related to missing data can be resolved in a timely fashion and to allow an assessment of the conformity of the available results with the sampling plan.

2.4.4. Member state reports

Upon receipt of a valid quarterly submission of data for the baseline survey, a summary report of the results will be sent by e-mail to the data provider. This will allow the data provider to confirm that the information submitted has been correctly received by EFSA. If requested, the report can also be sent to the NRL for confirmation of the submitted laboratory results. The report will include the following tables:

- number of samples taken by production area and month of sampling;
- number of samples taken by dispatch centre and month of sampling;

[18] https://dcf.efsa.europa.eu/dcf-war/dc
[19] http://www.w3.org/XML/
[20] http://www.w3.org/TR/xmlschema11-1/

- number of samples by production area, production system descriptors and species sampled;
- number of samples by dispatch centre, production and treatment information and species sampled;
- number of samples below LOQ and minimum, maximum, median and mean detectable genome copies for samples above LOQ for production areas NoV by genotype and for total NoV;
- number of samples below LOQ and minimum, maximum, median and mean detectable genome copies for samples above LOQ for dispatch centres by NoV genotype and for total NoV;
- proportion of samples < LOQ, 100, 200, 500, 1,000, 5,000, 10,000 and > 10,000 copies/g by production area classification and month of sampling;
- proportion of samples from batches < LOQ, 100, 200, 500, 1,000, 5,000, 10,000 and > 10,000 copies/gram by production area classification as indicated in traceability documents and month of sampling.

2.4.5. Production volumes

For the analysis of the baseline survey results, there is a requirement to adjust the reported results for batches by the production volumes in Europe for the duration of the survey. In order to obtain this data, the following information on production volumes should be submitted to the DCF upon completion of the survey. This information should be collected from all dispatch centres (not only those sampled) in each country.

Table 6: Description of variables to be reported for production volumes

Element Name	Data Type	M[(a)]	Controlled terminology	Description
PlantId	xs:string (250)	Y	Dispatch centre code (Appendix D)	Dispatch Centre Approval Number for the source of the batches
Country	xs:string (2)	Y	COUNTRY	Country where the dispatch centre is located
Y	xs:integer (4)	Y		Year of production
M	xs:integer (2)	Y		Month of production
batch	xs:integer (30)	Y		Number of batches produced in the dispatch centre in the month reported above
tonnage	xs:double			Production volume for the dispatch centre in the month reported above in kilograms

(a): 'Y' indicates this element is mandatory and must be completed

2.5. Plan of analysis

In order to provide a general description of the collected data in terms of sample size, a table containing the planned and achieved sample size for each country participating in the survey will be prepared for production areas and dispatch centres.

Exploratory analysis of all data collected will be carried out, considering simple statistics, such as mean, standard deviation, frequency tables, and graphs in order to provide a general overview of the data submitted.

Generalized linear (mixed) models will be fitted depending on the population structure to be analysed to estimate proportion of sample units as previously defined above the following thresholds (< LOQ, 100, 200, 500, 1,000, 5,000, 10,000 and > 10,000 copies/g) as well as 95% confidence intervals, considered as primary endpoint. Proportions will only be presented at a European level; no country specific analysis is planned.

The same type of model will be extended and used to estimate the potential effect of countries, time of sampling, norovirus genotype, classification of the production area (categories A, B and C) and other potential risk factors that could influence the proportion of sample units above the prespecified number of copies threshold. The obtained results will be shown using graphs, tables-containing estimated values of the parameters as well as standard error and significance level.

As the primary objective of the survey is not to assess specific risk factors effects, sparseness/separation could be an issue when carrying out the analysis. Ensoy et. al (2015) proposed a structure and harmonised guidance on how to deal with such issues, depending on the type of analysis performed, this will be used when analysing the Nov baseline survey data. Figure 5 presents the strategy that will be followed to deal with such issues.

Survival models considering censoring of the number of copies (considered as secondary endpoint) reported will be explored accounting for the risk factors previously mentioned. The distribution of number of copies at EU level will be fitted considering the nature of the outcome (log-normal, Gamma, Weibull, Exponential, etc.). The resulting best-fitted distribution can then be used to estimate the number of sample units below specific thresholds of number of copies.

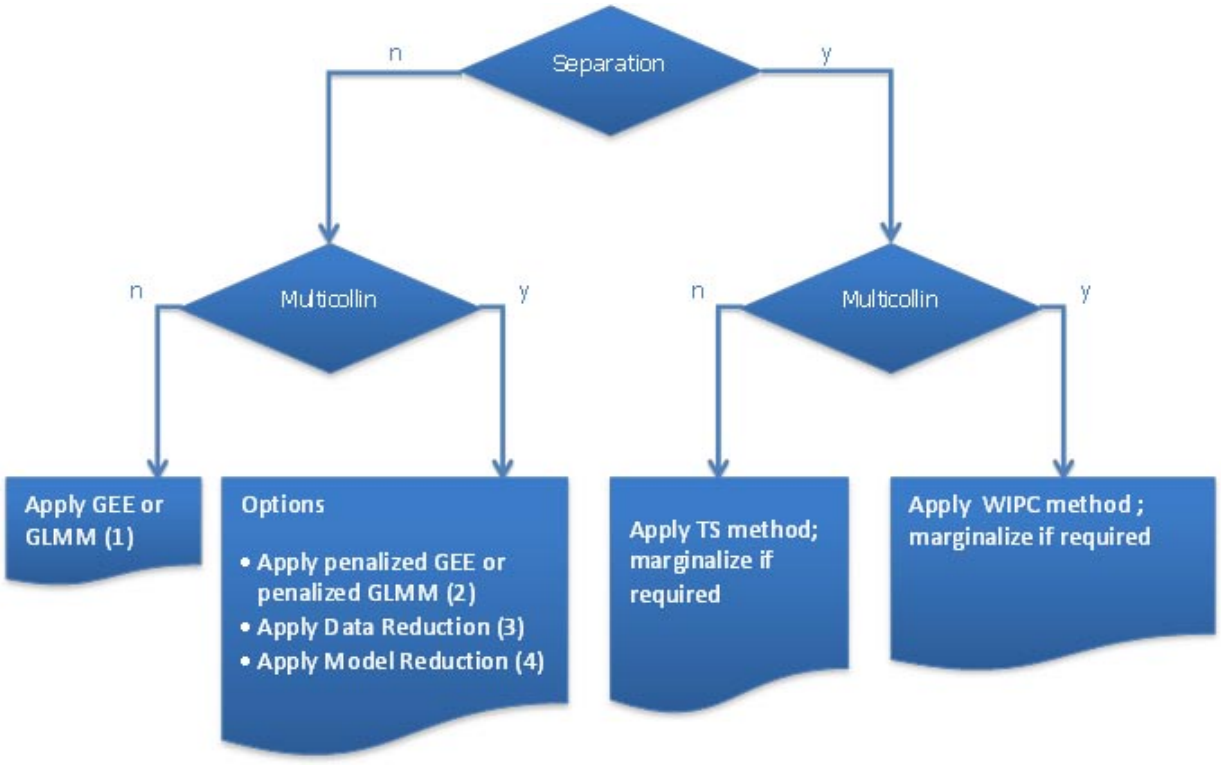

Figure 5: Decision tree for analysis of NoV baseline survey data in case of separation issues Ensoy et. al. (2015)

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Abbreviations

CA	Competent Authority
CEFAS	Centre for Environment, Fisheries & Aquaculture Science
DCF	data collection framework
EURL	European Union Reference Laboratory
LBM	Live Bivalve Molluscs
LOD	limit of detection
LOQ	limit of quantification
MS	Member State/s
NoV	Norovirus
NRL	National Reference Laboratory
PAFF	Standing Committee on Plants, Animals, Food and Feed
PCR	polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
SRS	simple random sampling schemes
XML	Extensible mark-up language

Appendix A – Oyster production figures

Table 7: Proportional allocation of dispatch centres to be sampled (dispatch centres currently active handling *C. gigas* and/or *O. edulis*

Country	Average (2009–2013) tonnes *C. gigas*[21])	Average (2009–2013) tonnes *O. edulis*[22])	Proportion of EU production	Total sales volume 1,000 tonnes[23] 2012
Croatia		40.7426	0.04%	
France	88,603	987.98	89.03%	118.4
Germany	82		0.08%	
Ireland	7,317.88	310.94	7.58%	7.4
Netherlands		140.14	0.14%	3.5
Portugal				0.8
Spain	648.811	760.1182	1.40%	
United Kingdom	1,635.72	97.97	1.72%	
Grand Total	**98,287**	**2,338**	**100.00%**	

[21] FAO fishstat http://www.fao.org/fishery/statistics/en
[22] see footnote 21
[23] http://stecf.jrc.ec.europa.eu/documents/43805/839433/2014-11_STECF+14-18+-+EU+Aquaculture+sector_JRC93169.pdf

Table 8: First sale/landings of oysters in 2014 (volume kg[(a)]) by month and country

	Month												
Country	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**10**	**11**	**12**	**Totals**
Denmark			9,671	24,360					30	8,570	24,712	15,996	83,339
France	3,005	1,864	2,092	1,061	1,130	953	1,132	2,250	1,566	3,804	19,773	15,827	54,457
Netherlands												11	11
Portugal	3,646	3,957	3,956	8,293	7,676	8,565	7,475	7,524	6,751	5,334	652	2,221	66,050
United Kingdom	878.5	931.5	100	1,597	1,801			764	382.4	187	26,165	921.7	33,728.1
Totals	**7,529.50**	**6,752.50**	**15,819**	**35,311**	**10,607**	**9,518**	**8,607**	**10,538**	**8,729.40**	**17,895**	**71,302**	**34,976.70**	**237,585**

(a): http://www.eumofa.eu/adhoc/topics/market_mon

Appendix B – Method specification for laboratory analysis

European Union Reference laboratory for monitoring bacteriological and viral contamination of bivalve molluscs

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Quantitative detection of norovirus genogroup I and genogroup II in oysters

Version 1

1. Introduction

Laboratories performing norovirus analysis for the European Food Safety Authority (EFSA) European Union (EU)-wide baseline survey in oysters are required to be designated by the Competent Authority (CA) of the Member State (MS). The analysis in each designated laboratory is required to be supervised by the National Reference Laboratory (NRL). This method specification has been compiled and agreed by the European Reference Laboratory (EURL)/EFSA technical working group comprised of representatives from the EURL and EFSA, and NRLs of France, Ireland, Germany, Denmark and Italy, and sets out the procedures that designated laboratories are required to follow in performing analysis of oysters submitted under this survey.

This method specification is based on, and is compliant with, ISO/DIS 15216-1; Microbiology of the food chain -- Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR -- Part 1: Method for quantification (the initial draft revision to the ISO method including validation data). Accordingly, there are some differences to the current published technical specification ISO/TS 15216-1:2013. Furthermore, ISO/DIS 15216-1 has now passed the ISO Enquiry Stage and is due to be revised into a final draft (FDIS) in response to Technical comments received by the voting National Standards bodies. Following approval, the FDIS will be published as an International Standard (projected publication date last quarter of 2016). Further modifications to this method specification may therefore be necessary to harmonise fully with the agreed text of the FDIS (and by extension the forthcoming published ISO) prior to the start of the baseline survey. However, it is anticipated that any such changes will be comparatively minor and will not affect laboratories' ability to undertake the analytical method. The EURL will circulate further information as it becomes available.

In this method, specification flexibility in reagents and processes is allowed consistent with the text of ISO/DIS 15216-1; however, more detailed methods as recommended by the EURL are included as appendices for information.

In addition to detailed methods in the appendices, the EURL will offer, on request, additional support to designated laboratories in the form of ready-to-use control materials (dsDNA and EC RNA for both norovirus genogroup I (GI) and genogroup II (GII), and mengo virus strain MC_0 for use as process control), and a spreadsheet for calculation of results from the raw data (C_q values). Use of control materials and the quantification spreadsheet provided by the EURL is not mandatory. In all cases, designated laboratories, prior to initiation of analysis, must produce a detailed bench protocol for use in their laboratory for the duration of the baseline survey, which is consistent with the text of this method specification, and agreed with their NRL. This protocol must cover all stages of the process including generation of control materials. It is the responsibility of the NRL to ensure that all laboratory protocols proposed in their MS are compliant with this method specification and to maintain electronic copies of these documents for future review.

3. Safety precautions

Standard microbiology safety precautions should be applied throughout. Laboratories should perform a full risk assessment before performing this procedure.

4. Equipment

- micropipettes;
- micropipette tips of a range of sizes, 1000 µl, 200 µl, 20 µl and 10 µl;
- pipette filler;
- pipettes of a range of sizes, 10 ml, 5 ml;
- vortex mixer;
- shaking incubator operating at 37°C and 320 rpm or equivalent;
- aspirator or equivalent apparatus for removing supernatant;
- waterbath capable of operating at 60°C or equivalent;

- bench centrifuge and rotor capable of running at 3,000 x g with capacity, for 15 or 50-ml tubes;
- microcentrifuge;
- balance;
- centrifuge and microcentrifuge tubes/bottles of a range of sizes, 1.5 ml, 15 ml, 50 ml, etc.;
- sterile shucking knife (for opening shellfish) or equivalent;
- rubber block for shucking (opening) shellfish or equivalent;
- scissors and forceps for dissecting shellfish or equivalent;
- heavy duty safety glove;
- sterile Petri dishes;
- razor blades;
- RNA extraction equipment suitable for extraction methods using silica and associated reagents. See Addendum 1 for illustrative details of RNA extraction apparatus as recommended by the EURL;
- PCR machine with real-time capacity capable of supporting hydrolysis probe (TaqMan®) chemistry;
- consumables for real-time PCR, e.g. optical plates and caps.

5. Reagents

5.1. Reagents used as purchased

- molecular biology grade water;
- tris base;
- EDTA;
- sodium chloride (NaCl);
- potassium chloride (KCl);
- disodium hydrogenphosphate (Na2HPO4);
- potassium dihydrogenphosphate (KH2PO4);
- proteinase K (30 U/mg);
- silica, lysis, wash and elution buffers for extraction of viral RNA. Reagents shall enable processing of 500 µl of supernatant, using lysis with a chaotropic buffer containing guanidine thiocyanate and using silica as the RNA-binding matrix. Following treatment of silica-bound RNA with wash buffer(s) to remove impurities, RNA shall be eluted in 100 µl elution buffer;
- the RNA preparation shall be of a quality and concentration suitable for the intended purpose. See Addendum 1 for illustrative details of RNA extraction reagents as recommended by the EURL;
- reagents for one-step real-time RT-PCR using hydrolysis probes (Taqman®). Reagents shall allow processing of 5 µl RNA in 25 µl total volume. They shall be sufficiently sensitive for the detection of levels of virus RNA as typically found in virus-contaminated oysters. See Addendum 3 for illustrative details of one-step real-time RT-PCR reagents as recommended by the EURL;
- primers and hydrolysis (Taqman®) probes for detection of norovirus GI and GII. Primer and probe sequences shall be published in a peer-reviewed journal and be verified for use against a broad range of strains of target virus. Primers shall target the ORF1/ORF2 junction of the genome. See Addendum 2 for illustrative details of primers and probes as recommended by the EURL;

- primers and hydrolysis (Taqman®) probes for detection of the process control virus. Primer and probe sequences shall be published in a peer-reviewed journal and be verified for use against the strain of process virus used. They shall demonstrate no cross-reactivity with norovirus genogroup I or genogroup II. See Addendum 2 for illustrative details of primers and probes as recommended by the EURL.

5.2. Prepared solutions/buffers

- **Proteinase K solution**

  Add 20 mg proteinase K (30 U/mg) to 200 ml water. Shake to dissolve then store in working aliquots at < -15ºC for a maximum of 6 months. Once defrosted store aliquots refrigerated and use within 1 week.

- **1 M Tris solution**

  Add 121 g Tris base to 1,000 ml water. Shake to dissolve, then adjust pH to 8.0 ± 0.2. Sterilise by autoclaving.

- **0.5 M EDTA solution**

  Add 186 g EDTA to 1,000 ml water. Shake to dissolve, then adjust pH to 8.0 ± 0.2. Sterilise by autoclaving.

- **TE buffer**

  Mix together 1 ml 1 M Tris solution, 200 µl 0.5 M EDTA solution and 100 ml water. Shake to mix. Alternatively use a commercial TE buffer preparation.

- **PBS buffer**

  Add 8 g sodium chloride, 0.2 g potassium chloride, 1.15 g disodium hydrogenphosphate and 0.2 g potassium dihydrogenphosphate to 1,000 ml water. Shake to dissolve, then adjust pH to 7.3 ± 0.2. Sterilise by autoclaving. Alternatively use a commercial PBS buffer preparation.

- **Real-time RT-PCR mastermixes for norovirus and process control virus.**

  Reagents shall be added in quantities as specified by the manufacturers to allow 20 µl mastermix per reaction in a 25 µl total volume. Optimal primer and probe concentrations shall be used after determination following the recommendations of the reagent manufacturers. See Addendum 3 for illustrative details of real-time RT-PCR mastermixes as recommended by the EURL.

  Note: Real-time RT-PCR mastermixes must be prepared no more than 24 h before use. Short-term storage (< 24 hours) at 2–6ºC is appropriate. Always prepare enough buffer for at least one reaction more than required (for larger preparations a greater number of excess reactions may be necessary).

5.3. Control materials

- **Process control virus**

  The virus selected for use as a process control shall be a culturable non-enveloped positive-sense ssRNA virus of a similar size to norovirus to provide a good morphological and physicochemical model. The process control virus shall exhibit similar persistence in the environment to norovirus. The virus shall be sufficiently distinct genetically from norovirus that real-time RT-PCR assays for norovirus and the process control virus do not cross react, and the process control virus shall not normally be expected to occur naturally in oysters.

  Process control virus stock shall be diluted by a minimum factor of 10 in a suitable buffer, e.g. PBS. This dilution shall allow for inhibition-free detection of the process control virus

genome using real-time RT-PCR, but still be sufficiently concentrated to allow reproducible determination of the lowest dilution used for the process control virus RNA standard curve. Split the diluted process control virus material into single use aliquots and store at -15°C or below. See Addendum 4 for illustrative details of the preparation of process control virus as recommended by the EURL.

- **Double-stranded DNA (dsDNA) control material.**

  Purified linear DNA molecules carrying the target sequence for each norovirus genogroup shall be used. The sequence of the DNA molecules shall be verified prior to use. The preparations shall not cause RT-PCR inhibition. The concentrations of each dsDNA stock in template copies per microlitre shall be determined using an appropriate method, e.g. spectrophotometry, fluorimetry or digital PCR, then the stock shall be diluted in a suitable buffer, e.g. TE buffer [NOTE: Do not use water], to a concentration of 1 × 104 to 1 × 105 template copies/µl. Split the diluted dsDNA control material into single use aliquots and store frozen at −15°C or below. See Addendum 5 for illustrative details of the preparation of dsDNA as recommended by the EURL.

- **External control RNA (EC RNA)**

  Purified ssRNA carrying the target sequence for each norovirus genogroup shall be used. They shall contain levels of contaminating target DNA no higher than 0.1% and shall not cause RT-PCR inhibition. The concentrations of each EC RNA stock in copies per microlitre shall be determined then the stock shall be diluted in a suitable buffer, e.g. TE buffer (NOTE: Do not use water), to a concentration of 1 × 104 to 1 × 105 template copies/µl. Split the diluted EC RNA preparation (EC RNA control material) into single use aliquots and store frozen at −15 °C or below. See Addendum 6 for illustrative details of the preparation of EC RNA as recommended by the EURL.

6. Method

Sample extraction and real-time RT-PCR shall be carried out in separate working areas or rooms. Particular care should be taken to ensure that positive control materials are not introduced into sample extraction areas.

6.1. Sampling and sample transport

Sampling and sample transport must be carried out in accordance with the requirements laid out in the EFSA Technical Specification for the Baseline Survey as set out elsewhere in this document.

6.2. Sample acceptance criteria and initial preparation

Sample processing must be initiated within 72 h of sample collection. If this is not possible due to, for example, delays during transportation, the sample should be regarded as unsatisfactory and analysis should not be carried out. Samples must be received in an intact food grade plastic bag and properly packed in a cool box with ice packs. Samples should be regarded as unsatisfactory and analysis should not be carried out if on receipt at the laboratory the sample is frozen, the container is leaking, the shellfish are covered in mud or immersed in water or mud/sand. Upon receipt in the laboratory, the internal air temperature of the transit container should be recorded. For samples where more than 4 h have elapsed between collection and receipt, the internal air temperature should be between 0 and 10°C. If the internal air temperature is greater than 10°C, the sample temperature should be measured; this should not exceed 10°C. For samples where less than 4 h have elapsed between collection and receipt, internal air temperature should be less than the temperature recorded at the time of sampling. Samples should be regarded as unsatisfactory and analysis should not be carried out if the temperature recorded does not meet the criteria specified. Choose oysters that are alive according to the following criteria:

- if any exposed flesh reacts to touch using a sterile shucking knife;
- if oysters open and close of their own accord;

- if a tap on the shell causes closing or movement;
- tightly closed shellfish.

Discard all dead shellfish and those with obvious signs of damage. Select a minimum of 10 animals. More than 10 shellfish can be used, if necessary, to produce the required quantity of digestive glands (> 2 g of digestive glands must be produced, and if possible > 4 g should be produced). If fewer than 10 live, undamaged animals are available, or if < 2 g digestive glands are produced, analysis should not be carried out.

Small amounts of mud and sediment adhering to the shell should be removed prior to opening by rinsing/scrubbing under cold, running tap water of potable quality. Shellfish should not be reimmersed in water as this may cause them to open. Weigh the selected oysters to determine the average weight of an individual oyster with shell in grams.

6.3. Dissection of digestive glands

Place the oyster on a rubber block or equivalent. Open the shells with a clean knife or equivalent. Ensure the hand holding the oyster is adequately protected, e.g. with a heavy-duty safety glove.

Dissect out the digestive glands using scissors and forceps (or equivalent tools).

Transfer to a clean petri dish and chop finely with a razor blade (or equivalent).

Transfer a 2 g portion of chopped glands into a centrifuge tube. Process immediately, store at 4°C for up to 24 h, at -15°C or below for up to 6 months, or at -70°C or below for longer periods. The remaining chopped glands can be stored in 2 g aliquots at -15°C or below for up to 6 months, or at -70°C or below for longer periods.

6.4. Virus extraction

Immediately before any batch of samples is processed, pool together sufficient aliquots of process control virus material for use with all samples (allow 10 µl per sample plus 25 µl excess).

Retain a 20 µl subsample of pooled material for RNA extraction and preparation of the standard curve. Store at 4ºC for a maximum of 24 h or at -15ºC or below for longer periods.

Add 10 µl of process control virus material to the 2 g portion of chopped glands produced in Section 7.3. Immediately add 2 ml of proteinase K solution and mix well.

Incubate at 37°C in a shaking incubator or equivalent at 320 rpm for 60 min.

Carry out a secondary proteinase K incubation by placing the tube in a water bath or equivalent at 60ºC for 15 min.

Centrifuge at 3,000 x *g* for 5 min, decant the supernatant, measure and record the volume in millilitre and retain for downstream testing (RNA extraction). Process immediately, store at 4°C for up to 24 h, at -15°C or below for up to 6 months, or at -70°C or below for longer periods.

6.5. RNA extraction

Note: For every set of samples, a negative extraction control consisting of 500 µl water should be extracted in parallel.

Extract RNA from 500 µl of supernatant (retained from Section 7.4) for each sample using an appropriate method including lysis with a chaotropic buffer containing guanidine thiocyanate and using silica as the RNA-binding matrix as described in Section 6.1.

Elute purified RNA into 100 µl of elution buffer and retain for real-time RT-PCR analysis. Extracted RNA shall be processed immediately, stored at 4°C for up to 24 h, at -15°C or below for up to 6 months, or at -70°C or below for longer periods.

Note: Freeze-thaw cycles of stored RNA should be minimised.

See Addendum 1 for illustrative details of an RNA extraction method as recommended by the EURL.

6.6. Real-time RT-PCR analysis – general requirements

Real-time RT-PCR analysis for all targets need not be carried out on the same plate – however, the following restrictions must be observed;

Full sets of norovirus GI or GII assay control reactions (dsDNA dilution series, EC RNA and water only) should be used for every plate where sample RNA is assayed for that genogroup.

Full sets of process control virus assay control reactions (RNA dilution series from all relevant batches of process control virus material and water only) must be included on every plate where sample RNA is assayed for process control virus.

As results generated using 10^{-1} sample, RNA are only used in the event that RT-PCR inhibition is unacceptable for undiluted sample RNA (Section 7.10), it is permitted for laboratories to omit 10^{-1} sample RNA from the initial analysis of norovirus GI, norovirus GII and process control virus. In this case, where undiluted sample RNA provides an unacceptable RT-PCR inhibition, samples should subsequently be retested for any/all affected genogroups and process control virus using 10^{-1} sample RNA. Undiluted sample RNA does not need to be retested at this stage.

6.7. Real-time RT-PCR plate set-up – analysis of norovirus GI and/or GII

Note: This section describes plate set-up for a <u>single</u> genogroup.

Before starting 96-well real-time PCR plate preparation, prepare 10^{-1} dilutions of each sample RNA in water. (NOTE: If diluted RNA is not included in the initial analysis as described in Section 7.6, this step does not need to be carried out and wells using 10^{-1} sample RNA can be omitted).

Prepare 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of target dsDNA control material in a suitable buffer (e.g. TE buffer) (NOTE: Do not use water).

For each sample, prepare:

- two wells of an optical plate with 5 µl of undiluted sample RNA;
- two wells with 5 µl of 10^{-1} sample RNA;
- one well with 5 µl of undiluted sample RNA and 1 µl of undiluted EC RNA;
- one well with 5 µl of 10^{-1} sample RNA and 1 µl of undiluted EC RNA.

For the EC RNA control, prepare:

- one well with 5 µl of water and 1 µl of undiluted EC RNA.

For the dsDNA standard curve, prepare:

- two wells with 5 µl of undiluted dsDNA;
- two wells with 5 µl of 10^{-1} dsDNA;
- two wells with 5 µl of 10^{-2} dsDNA;
- two wells with 5 µl of 10^{-3} dsDNA;
- two wells with 5 µl of 10^{-4} dsDNA.

For negative controls, prepare:

- two wells with 5 µl of water;
- two wells with 5 µl of negative extraction control RNA.

Add 20 µl of the relevant real-time RT-PCR mastermix to each well (mastermix may also be added to all relevant wells before addition of template material).

6.8. Real-time RT-PCR plate set-up – analysis of process control virus

Before starting 96-well real-time PCR plate preparation, prepare 10^{-1} dilutions of each sample RNA in water. (NOTE: If diluted RNA is not included in the initial analysis as described in Section 7.6, this step does not need to be carried out and wells using 10^{-1} sample RNA can be omitted).

For each batch used with the samples under test, add 10 µl of process control virus material to a separate 500 µl portion of water. Extract and store RNA for each batch using the same method and conditions applied to the test samples.

For each batch of process control virus material extracted, prepare 10^{-1}, 10^{-2} and 10^{-3} dilutions of process control virus RNA in a suitable buffer (e.g. TE buffer) (NOTE: Do not use water).

For each sample, prepare:

- one well with 5 µl of undiluted sample RNA;
- one well with 5 µl of 10^{-1} sample RNA.

For the process control virus RNA standard curve, prepare:

- one well with 5 µl of undiluted process control virus RNA;
- one well with 5 µl of 10^{-1} process control virus RNA;
- one well with 5 µl of 10^{-2} process control virus RNA;
- one well with 5 µl of 10^{-3} process control virus RNA.

For negative controls, prepare:

- one well with 5 µl of water;
- one well with 5 µl of negative extraction control RNA.

Add 20 µl of process control virus real-time RT-PCR mastermix to each well (mastermix may also be added to all relevant wells before addition of template material).

Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + GI EC RNA	Test sample (-1) + GI EC RNA	H2O + GI EC RNA	-ve extraction control	-ve extraction control	H_2O	H2O	
GI dsDNA (undiluted)	GI dsDNA (undiluted)	GI dsDNA (-1)	GI dsDNA (-1)	GI dsDNA (-2)	GI dsDNA (-2)	GI dsDNA (-3)	GI dsDNA (-3)	GI dsDNA (-4)	GI dsDNA (-4)		
Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + GII EC RNA	Test sample (-1) + GII EC RNA	H2O + GII EC RNA	-ve extraction control	-ve extraction control	H_2O	H2O	
GII dsDNA (undiluted)	GII dsDNA (undiluted)	GII dsDNA (-1)	GII dsDNA (-1)	GII dsDNA (-2)	GII dsDNA (-2)	GII dsDNA (-3)	GII dsDNA (-3)	GII dsDNA (-4)	GII dsDNA (-4)		
Test sample (undiluted)	Test sample (-1)	Process control virus RNA (undiluted)	Process control virus RNA (-1)	Process control virus RNA (-2)	Process control virus RNA (-3)	-ve extraction control	H_2O				

Figure 6: Example plate layout (single sample – all assays on one plate) including wells with 10^{-1} sample RNA

NOTE: This layout includes wells with 10^{-1} sample RNA, however, these can be omitted from the initial analysis as described in Section 7.6

Norovirus GI assay
Norovirus GII assay
Process control virus assay

5 µl RNA (+/- 1 µl EC RNA) and 20 µl mastermix per well

Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + GI EC RNA	Test sample (-1) + GI EC RNA	H2O + GI EC RNA	-ve extraction control	-ve extraction control	H_2O	H2O	
GI dsDNA (undiluted)	GI dsDNA (undiluted)	GI dsDNA (-1)	GI dsDNA (-1)	GI dsDNA (-2)	GI dsDNA (-2)	GI dsDNA (-3)	GI dsDNA (-3)	GI dsDNA (-4)	GI dsDNA (-4)		
Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + GII EC RNA	Test sample (-1) + GII EC RNA	H2O + GII EC RNA	-ve extraction control	-ve extraction control	H_2O	H2O	
GII dsDNA (undiluted)	GII dsDNA (undiluted)	GII dsDNA (-1)	GII dsDNA (-1)	GII dsDNA (-2)	GII dsDNA (-2)	GII dsDNA (-3)	GII dsDNA (-3)	GII dsDNA (-4)	GII dsDNA (-4)		
Test sample (undiluted)	Test sample (-1)	Process control virus RNA (undiluted)	Process control virus RNA (-1)	Process control virus RNA (-2)	Process control virus RNA (-3)	-ve extraction control	H_2O				

Figure 7: Example plate layout (single sample – all assays on one plate) omitting wells with 10^{-1} sample RNA

NOTE: This layout omits wells with 10^{-1} sample RNA as described in Section 7.6

Norovirus GI assay
Norovirus GII assay
Process control virus assay

5 µl RNA (+/- 1 µl EC RNA) and 20 µl mastermix per well

6.9. Real-time RT-PCR assay run parameters

Subject the plate to a reaction cycle including an initial stage for reverse transcription and at least 45 cycles of PCR using a real-time RT-PCR machine. The duration and temperatures of each stage (reverse transcription, RT deactivation, denaturation, annealing and extension) depends on the reagents used; they shall be based on the manufacturer's recommendations, but can be further optimised.

For real-time RT-PCR machines where the user can set the point of fluorescence data collection, this shall be set at the end of the extension stage.

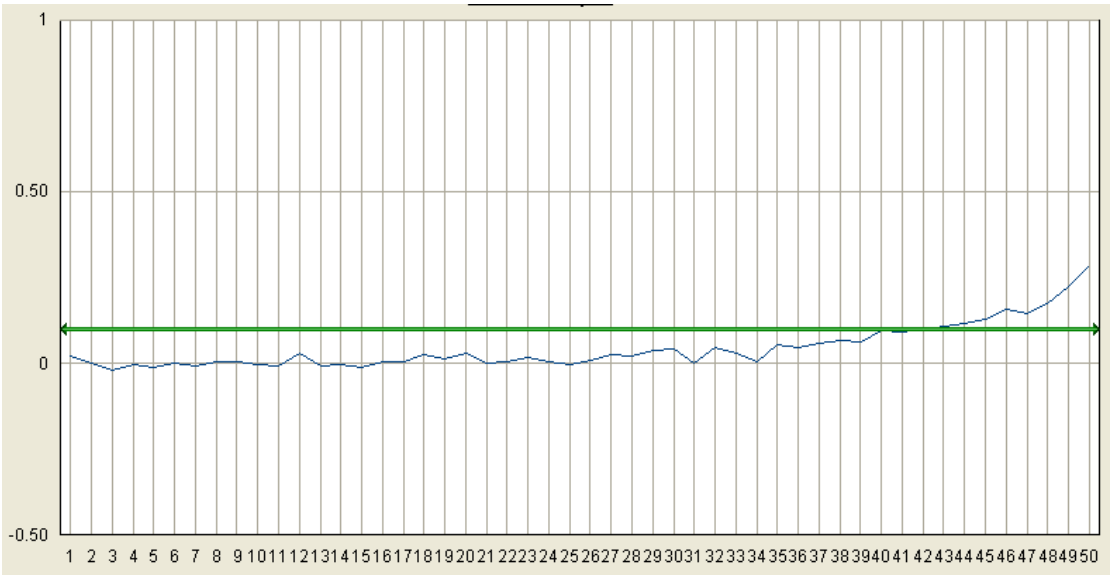
See Addendum 3 for illustrative details of an amplification method as recommended by the EURL.

6.10. Analysis of results

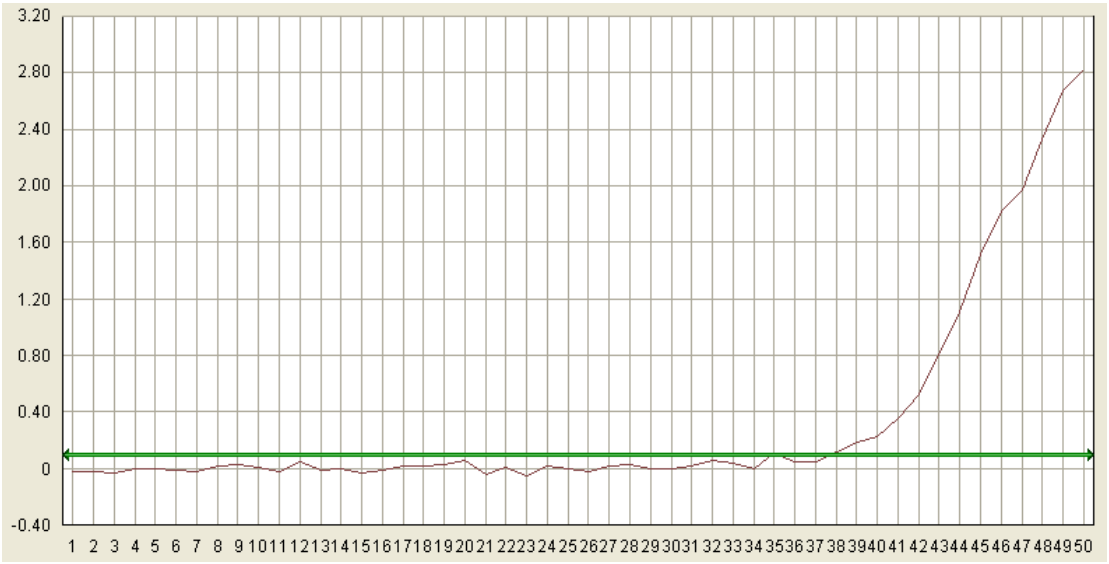
Note: The EURL will provide a calculation spreadsheet for this method on its website to support laboratories carrying out the analysis of results; this will make all necessary calculations based on the input C_q values. Its use is not mandatory however.

Analyse the amplification plots using the approach recommended by the manufacturer of the real-time PCR machine. The threshold should be set so that it crosses the area where the amplification plots (logarithmic view) are parallel (the exponential phase).

Check all amplification plots to identify false positive results (reactions with C_q values not associated with exponential amplification) caused by high or uneven background signal. Results for any wells affected in this way should be regarded as negative. An example is provided in the image below.



Check all amplification plots to identify true positive plots where the recorded C_q value is significantly distorted by high or uneven background signal. Approximate correct C_q values should be noted (in addition to the recorded value) for any wells affected in this way. Corrected C_q values should be used for all quantity calculations.



For example, in this case the recorded Cq value was 34.92, however, it should be noted by the laboratory that the correct figure should be, for example, 38.

Each control (dsDNA, EC RNA, process control virus RNA) has an expected valid value or range of values. If the observed result for any control is different from the expected value, samples may require retesting.

Negative controls (water and negative extraction control) shall always be negative; if positive results occur in these controls, then any samples giving positive results shall be retested.

Check Cq values of all standard curves for any points that do not fall close to the line of best fit. These Cq values should not be incorporated into standard curve calculations. No more than two such outlying Cq values shall be removed per series and values from a minimum of three (process control virus RNA) or four dilutions (dsDNA) must be included.

Use the remaining Cq values of each dilution series to create standard curves for each control by plotting the Cq values obtained against log10 concentration (e.g. log10 copies per microlitre target dsDNA) to determine r2, slope and intercept parameters. Do not average Cq values from duplicate reactions prior to plotting.

Curves with r2 values of < 0.980, or where the slope is not between -3.10 and -3.60 (corresponding to amplification efficiencies of ~90–110%), should not be used for calculations.

Use the Cq value for the undiluted sample RNA + EC RNA well to determine the RT-PCR inhibition levels for each sample and each norovirus genogroup by reference to the Cq value of the water + EC RNA well and the slope of the dsDNA standard curve as follows:-

$$\text{RT-PCR inhibition} = (1\text{-}10^{(\Delta C_q/\text{slope})}) \times 100\%$$

where ΔC_q = C_q value [sample RNA + EC RNA] - C_q value [water + EC RNA]

A sample (+ EC RNA) producing the same Cq values as undiluted EC RNA will have an RT-PCR inhibition level of 0%.

If the RT-PCR inhibition level is < 75% results for the undiluted RNA should be used for that sample and target. If the RT-PCR inhibition level is > 75%, repeat calculation with the 10^{-1} sample RNA + EC RNA wells for the same target (or repeat PCR using 10^{-1} sample RNA if this is not included in the initial analysis as described in Section 7.6). If the RT-PCR inhibition level using the 10^{-1} RNA is < 75%, results for the 10^{-1} RNA should be used for that sample and target. If RT-PCR inhibition levels for both undiluted and 10^{-1} sample, RNA are > 75% results are not valid and the sample should be retested.

Use the Cq value for the process control virus assay from the test sample RNA well (undiluted or 10^{-1} dependent on the RT-PCR inhibition results; see above) to estimate process control virus recovery by reference to the process control virus RNA standard curve as follows (if 10^{-1} sample RNA results are used multiply by 10 to correct for the dilution factor):

$$\text{Process control virus recovery} = 10^{(\Delta Cq/\text{slope})} \times 100\%$$

where ΔC_q = C_q value [sample RNA] – C_q value [undiluted process control virus RNA]

To determine the extraction efficiency, divide the process control virus recovery by 0.5 and multiply by the total measured volume of supernatant in millilitre (as determined in Section 7.4). Where the extraction efficiency is < 1% sample, results are not valid and the sample should be retested.

For a sample with acceptable RT-PCR inhibition and extraction efficiency results, if one or more replicate results from the sample RNA only wells (undiluted or 10^{-1} dependent on the RT-PCR inhibition results; see above) for a given norovirus genogroup are positive, the overall result for that sample will be regarded as positive. If both replicate results are negative, then the overall result for that sample will be regarded as not detected.

For each positive sample and norovirus genogroup, take the Cq values for the sample RNA only wells (undiluted or 10^{-1} dependent on the RT-PCR inhibition results; see above) and use these to calculate concentrations (in detectable virus genome copies/µl RNA) for each replicate by reference to the relevant dsDNA standard curve as follows:-

$$\text{concentration} = 10^{(\Delta Cq/\text{slope})}$$

where ΔC_q = C_q value [sample RNA] – standard curve intercept

Negative replicates should be given a concentration of zero copies/µl RNA. For each sample, calculate the average of the concentrations for both replicates.

Multiply this value by 200 (undiluted RNA) or 2,000 (10^{-1} RNA) then multiply by the total volume of supernatant in millilitre (as determined in Section 7.4) to calculate the number of detectable virus genome copies in the entire sample.

To obtain the concentration of the relevant norovirus genogroups in detectable virus genome copies per gram, divide the number of genome copies in the entire sample by the starting weight (2 g) of the sample.

7. Retesting

Where a sample provides an unacceptable RT-PCR inhibition level or extraction efficiency, or where control results indicate other problems with analysis, the samples should be retested according to the criteria outlined below.

A sample that fails to provide an acceptable RT-PCR inhibition level or extraction efficiency on two occasions does not need to be subjected to further retesting. In this case, results for the affected sample and genogroup shall be reported as no result, unless an otherwise valid positive result is obtained on at least one of the two testing occasions, in which case the result shall be regarded as positive, not quantifiable.

In all cases, test sample materials used for the retest must have been stored according to the relevant part of this protocol.

The following provides a list of situations where retesting may be required and recommendations for the appropriate procedure to undertake on retesting:

For samples where results are affected by problems with RT- PCR, e.g.:

- positive results in water only wells;
- negative or unexpected results with dsDNA or EC RNA controls;
- high background/low signal in general.

Retest using stored RNA (Section 7.5). Test only those assays where results have been affected. Where samples are retested for process control virus, the appropriate batch of process control virus RNA must also be tested.

For samples where negative extraction controls are contaminated (and negative RT-PCR controls – water only – are not contaminated):

Retest using stored digestive glands (Section 7.3) provided that 2 g stored glands are available, otherwise retest using stored supernatant (Section 7.4). Only affected genogroups need to be tested, however, full process control virus controls to enable assessment of extraction efficiency specifically for the retest must be used.

For samples where extraction efficiency is below the acceptable threshold:

Retest using stored digestive glands (Section 7.3) provided that 2g stored glands are available; otherwise retest using stored supernatant (Section 7.4).

For samples where RT-PCR inhibition level is above the acceptable threshold (after testing undiluted sample RNA, in the case that 10^{-1} sample RNA has not been included in the initial analysis as described in Section 7.6):

Retest using stored RNA (Section 7.5). Prepare a 10^{-1} dilution prior to analysis, and do not repeat analysis using undiluted RNA. Test only those assays where results have been affected.

For samples where RT-PCR inhibition level is above the acceptable threshold (after testing 10^{-1} sample RNA):

Retest using stored digestive glands (Section 7.3) provided that 2 g stored glands are available, otherwise retest using stored supernatant (Section 7.4). Only affected genogroups need to be tested, however, full process control virus controls to enable assessment of extraction efficiency specifically for the retest must be used.

8. Sharing of analysis between multiple laboratories

For practical purposes in some MSs, it may be helpful if different stages of the analysis of samples can be carried out in different laboratories. This is permitted with the approval of the NRL, provided that:

- the overall protocol is consistent with this method specification;
- transportation arrangements must ensure that during transfer between laboratories intermediate test sample materials (e.g. digestive glands, supernatant or RNA) do not exceed either the time or temperature limits detailed within this method specification.

9. Two-stage PCR analysis

For practical purposes in some MSs, it may be helpful if real-time RT-PCR analysis can be carried out in two stages, including an initial presence/absence screen, followed by quantification of positive samples. This is permitted with the approval of the NRL. In this case, analysis at each stage should be carried out according to Sections from 7.6 to 7.10 and 8.0 with the following exceptions:

- a dsDNA standard curve shall not be used during the presence/absence screen, however, all other sample and control reactions as detailed in Sections 7.7 and 7.8 must be included;

- determination of RT-PCR inhibition and extraction efficiency for each sample and assessment of whether retesting is necessary shall be carried out as part of the presence/absence screen and shall not be repeated during the quantification stage;
- to control for RT-PCR inhibition at the presence/absence screen stage, the method from ISO/TS 15216-2 shall be used; if the C_q value of the undiluted sample RNA + EC RNA well is < 2.00 greater than the C_q value of the water + EC RNA well, results for the undiluted RNA shall be used for that sample. If the Cq value of the undiluted sample RNA + EC RNA well is ≥2.00 greater than the Cq value of the water + EC RNA well, repeat the comparison with the 10^{-1} sample RNA + EC RNA well;
- if the Cq value of the 10^{-1} sample RNA + EC RNA well is < 2.00 greater than the Cq value of the water + EC RNA well, results for the 10^{-1} RNA shall be used for that sample. If the Cq value of the 10^{-1} sample RNA + EC RNA well is ≥2.00 greater than the Cq value of the water + EC RNA well, the results are not valid and the sample requires retesting;
- at the quantification stage, in addition to the dsDNA standard curve, two wells with 5 µl of water shall be used as the negative control, and one well with 5 µl of water and 1 µl of undiluted EC RNA shall be used as a generalised positive control for RT-PCR. Other controls shall be omitted;
- a sample that provides a positive result at the presence/absence screen stage, but does not provide a positive result at the quantification stage shall be regarded as positive; not quantifiable;
- where the two stages of the PCR analysis are carried out at different laboratories transportation arrangements must ensure that during transfer between laboratories the RNA does not exceed either the time or the temperature limits detailed within this method specification;

10. Reporting of results

For each sample and each genogroup, the following information will be collected, using a common result reporting system, which will be maintained by EFSA:

- average weight per oyster (with shells);
- date of completion of analysis;
- result (positive/ not detected/ no result);
- for positive results, whether the sample was quantifiable or not quantifiable (as described in Sections 8 and 10);
- for quantifiable positive results, the quantity determined in copies/g; this figure should not be censored according to any limit of quantification;
- for positive not quantifiable results, whether this was due to failure to provide an acceptable RT-PCR inhibition level or extraction efficiency on two occasions or failure to detect the target during the quantification stage in a sample that was positive during the presence/absence screen stage where two-stage PCR as described in Section 10 is used;
- the limit of quantification (LOQ) that applies to the result;
- the limit of detection (LOD) that applies to the result where this is available.

11. Sample archiving

An archive of residual digestive glands, supernatant and RNA for all samples must be retained for each MS by the NRL or a single laboratory designated by the NRL for at least 2 years following the completion of the survey. Materials must be retained within the temperature limits described elsewhere in this method specification. Use of archived materials for alternative purposes is only allowed with the agreement of the relevant NRL and CA.

Addendum 1: RNA extraction using the Biomerieux NucliSens® System (informative)

Equipment

- NucliSens miniMAG magnetic rack. **BioMerieux**[24]**,** cat number; 200299.
- NucliSens miniMAG instrument. **BioMerieux**[25]**,** cat number; 200305.
- 1.5 ml tubes with screw caps suitable for use with the miniMAG/easyMAG extraction systems.
- Thermoshaker operating at 60ºC and 1400 rpm or equivalent.

Reagents

- NucliSens magnetic extraction reagents. **BioMerieux**[26]**,** cat numbers; 200293, etc.
- NucliSens lysis buffer. **BioMerieux**[27]**,** cat numbers; 284135, 280134, etc.

Method

For each test sample, add 2 ml of NucliSens lysis buffer to a tube. Add 500 µl of supernatant produced in Section 7.4 and mix by vortexing briefly.

Incubate for 10 min at room temperature.

Add 50 µl of well-mixed magnetic silica solution to the tube and mix by vortexing briefly.

Incubate for 10 min at room temperature.

Centrifuge for 2 min at 1,500 x *g* then carefully discard supernatant by, for example, aspiration.

Add 400 µl wash buffer 1 and resuspend the pellet by pipetting/vortexing.

Transfer suspension to a 1.5 ml screw-cap tube. Wash for 30 s using the automated wash steps of the miniMAG/easyMAG extraction systems or by vortexing. After washing, allow silica to settle using magnet of the miniMAG/easyMAG extraction system. Discard supernatant by, for example, aspiration.

Separate tubes from magnet, then add 400 µl wash buffer 1. Resuspend pellet, wash for 30 s, allow silica to settle using magnet then discard supernatant.

Separate tubes from magnet, then add 500 µl wash buffer 2. Resuspend pellet, wash for 30 s, allow silica to settle using magnet then discard supernatant. Repeat.

Separate tubes from magnet, then add 500 µl wash buffer 3. Wash for 15 s, allow silica to settle using magnet then discard supernatant.

Note: Samples should not be left in wash buffer 3 for longer than strictly necessary.

Add 100 µl elution buffer, cap tubes and transfer to thermoshaker or equivalent.

Incubate for 5 min at 60ºC with shaking at 1,400 rpm.

Place tubes in magnetic rack and allow silica to settle, then transfer eluate to a clean tube. Process immediately, store at 4°C for up to 24 h, at -15°C or below for up to 6 months, or at -70°C or below for longer periods.

[24] http://www.biomerieux.com/
[25] see footnote 24
[26] see footnote 24
[27] see footnote 24

Addendum 2: Primer and probe sequences (informative)

Norovirus GI

QNIF4 (FW):	CGC TGG ATG CGN TTC CAT	[da Silva et al., 2007]
NV1LCR (REV):	CCT TAG ACG CCA TCA TCA TTT AC	[Svraka et al., 2007]
TM9 (PROBE):	TGG ACA GGA GAT CGC	[Hoehne & Schreier, 2006]

Probe labelled 5' FAM, 3' MGBNFQ

Norovirus GII

QNIF2 (FW):	ATG TTC AGR TGG ATG AGR TTC TCW GA	[Loisy et al., 2005]
COG2R (REV):	TCG ACG CCA TCT TCA TTC ACA	[Kageyama et al., 2003]
QNIFS (PROBE):	AGC ACG TGG GAG GGC GAT CG	[Loisy et al., 2005]

Probe labelled 5' FAM, 3' 6-carboxy-tetramethylrhodamine (TAMRA)

Mengo virus

Mengo 110 (FW):	GCG GGT CCT GCC GAA AGT	[Pintó et al., 2009]
Mengo 209 (REV):	GAA GTA ACA TAT AGA CAG ACG CAC AC	[Pintó et al., 2009]
Mengo 147 (PROBE):	ATC ACA TTA CTG GCC GAA GC	[Pintó et al., 2009]

Probe labelled 5' FAM, 3' MGBNFQ

pTAG (for pGEM series plasmids)

pTAG 5:	GCT ATG ACC ATG ATT ACG CCA A	[Maguire et al., 1999]
pTAG 3:	TGT AAA ACG ACG GCC AGT GAA	[Maguire et al., 1999]

Addendum 3: Real-time RT-PCR mastermixes and cycling parameters (informative)

Real-time (Taqman®) RT-PCR mastermixes are prepared using the RNA Ultrasense One-step qRT-PCR system. Thermo Scientific catalogue number; 11732927.[28]

Reagent	Final concentration (in 25 µl)	Volume per reaction (µl)
5× Ultrasense reaction mix	1×	5
FW Primer	0.5 pmol/µl	As required
REV Primer	0.9 pmol/µl	As required
Probe	0.25 pmol/µl	As required
ROX reference dye (50×)	As require[(a)]	As required
RNA Ultrasense enzyme mix	—	1.25
Water	—	As required
Total volume	—	20

[(a)] With Applied Biosystems real-time RT-PCR machines, ROX shall be used at 1× concentration; for the Stratagene MX3000, ROX can be either used at 0,1× concentration, or omitted from the mastermix. For other machines, consult the manufacturer's instructions.

Cycling parameters

Step description		Temperature and time	Number of cycles
RT		55°C for 1 h	1
Preheating		95°C for 5 min	1
Amplification	Denaturation	95°C for 15 s	45
	Annealing-extension	60°C for 1 min	
		65°C for 1 min	

Addendum 4: Growth of mengo virus strain MC_0 for use as a process control (informative)

Note: For preparation of this, control material laboratories will require cell culture facilities including incubator(s), preferably with controllable CO_2 levels, cell culture consumables (flasks, etc.) and media.

The EURL will, on request, provide ready-to-use mengo virus process control material (prepared as detailed below) to designated laboratories to cover the analysis of samples during the EFSA baseline survey.

Mengo virus strain MC_0 (ATCC VR-1597) should be used unless proscribed by e.g. GMO regulations. In this case, wild-type mengo virus (ATCC VR-1598) can be used. Mengo virus should best be grown in a 5% CO_2 atmosphere (with open vessels) or an uncontrolled atmosphere (closed vessels) on 80–90% confluent monolayers of HeLa cells (ATCC CCL-2). Recommended cell culture medium for this cell line is:

Eagle's minimum essential medium with

2mM L-glutamine

Earle's BSS, adjusted to

1.5 g/l sodium bicarbonate

[28] http://www.thermoscientific.com/content/dam/tfs/SDG/MBD/MBD%20Marketing%20Material/Food/LifeTech-Micro-Product%20List-EN.pdf

0.1 mM non-essential amino acids

1.0 mM sodium pyruvate

1% streptomycin/penicillin

10% (growth) or 2% (maintenance) foetal bovine serum

Alternatively, virus can be grown on FRhK-4 cells (ATCC CRL-1688). Recommended cell culture medium for this cell line is:

Dulbecco's modified Eagle's medium with

4mM L-glutamine, adjusted to

1.5 g/l sodium bicarbonate

4.5 g/l glucose

1% streptomycin/penicillin

10% (growth) or 2% (maintenance) fetal bovine serum

To prepare mengo virus for process control, freeze and thaw a culture flask in which at least 75% cytopathic effect (CPE) has been reached, centrifuge flask contents at 3,000 x g for 10 min to clarify and retain supernatant. Dilute by a minimum factor of 10x in sample buffer, e.g. PBS, split into single use aliquots and store frozen at -80°C. This dilution must allow for inhibition-free detection of the process control virus genome using real-time RT-PCR but still be sufficiently concentrated to allow reproducible determination of the lowest dilution used for the process control virus RNA standard curve.

Addendum 5: Generation of double-stranded DNA (dsDNA) control stocks (informative)

Note: For preparation of these control materials, laboratories will require capabilities for transformation and growth in solid and liquid media of *E.coli*, capabilities or kits for plasmid preparation, conventional PCR and purification of DNA from reaction mixes (in addition to the listed products) and a spectrophotometer capable of measuring at 260 and 280 nm. Special care shall be taken to separate work areas used for generation of dsDNA control stocks from those used for sample extraction.

The EURL will, on request, provide ready-to-use dsDNA control material (prepared as detailed below) to designated laboratories to cover the analysis of samples during the EFSA baseline survey. However, please note that control material supplied by the EURL or generated using the method below will not work with all permissible primer/probe sets. If primer and probe sets different to those illustrated in Addendum 2 are used, it is the responsibility of the designated laboratory and the relevant NRL to ensure that dsDNA control material suitable for the primer/probe set selected is used.

Norovirus control plasmids used by the EURL were developed by Dr Françoise S. Le Guyader (Le Guyader et al., 2009). For norovirus, GI and GII control plasmids were separately constructed by ligating the target DNA sequence into the pGEM-3Zf(+) vector at a *Sma*I restriction site such that in each case the target sequence was downstream of a promoter sequence for the T7 RNA polymerase. The EURL can supply starter aliquots of these plasmids to laboratories upon request.

The plasmid should be transformed and maintained in, and purified from, *E. coli* cells using standard molecular and microbiology techniques. Following purification of plasmid by e.g. commercial miniprep, **linear** DNA molecules suitable for use as quantification controls can be generated by PCR amplification of an amplicon spanning the target region using a dilute solution of the plasmid as template and the pTAG 5 and pTAG 3 primer set (see Addendum 2 for primer sequences).

The linear dsDNA control should be verified by sequencing. Expected sequences (virus-derived sequences in bold) are:

Norovirus GI

TGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTCGAGCTCGGTACCC**CGCTGGATGCGCTTCCATGACCTCGGATTGTGGACAGGAGATCGCGATCTTCTGCGGATCCGAATTCGTAAATGATGATGGCGTCTAAGG**GGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGC

Norovirus GII

TGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTCGAGCTCGGTACCC**ATGTTCAGATGGATGAGATTCTCAGATCTGAGCACGTGGGAGGGCGATCGCAATCTGGCTCGGATCCCCAGCTTTGTGAATGAAGATGGCGTCGA**GGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGC

Following generation of linear control DNA, a small amount should be cleaned up using, for example, a commercial PCR purification kit. The concentration of DNA can then be calculated using spectral absorption at 260 nm (spectral absorption at 280 nm should also be carried out to monitor the purity of the DNA preparation, highly pure DNA should have an A260/280 ratio of around 1.8).

Multiplication of the A260 value by 5×10^{-8} (and by any dilution factor involved) will give the concentration of DNA in g/µl.

Divide this number by the mass in grams of a single dsDNA molecule to calculate the concentration of DNA in copies/µl (the mass of an individual dsDNA molecule is calculated by multiplying the length in bp by 607.4 (the molecular weight of an average bp) and dividing by the Avogadro constant (6.02×10^{23}).

For the pTAG, PCR products amplified from the plasmids used by the EURL the masses are as follows:

Norovirus GI	2.45×10^{-19}g	(242 bp)
Norovirus GII	2.50×10^{-19}g	(247 bp)

The preparation of linear dsDNA should then be diluted with a suitable buffer (e.g. TE buffer) (NOTE: do not use water) to a concentration of approximately 1×10^4–1×10^5 copies/µl, and frozen in single use aliquots.

Addendum 6: Generation of external control RNA (EC RNA) control stocks (informative)

Note: For preparation of these control materials, laboratories will require capabilities for transformation and growth in solid and liquid media of *E. coli*, capabilities or kits for plasmid preparation, purification of DNA from reaction mixes (in addition to the listed products) and a spectrophotometer capable of measuring at 260 nm. Special care shall be taken to separate work areas used for generation of dsDNA control stocks from those used for sample extraction.

The EURL will, on request, provide ready-to-use EC RNA control material (prepared as detailed below) to designated laboratories to cover the analysis of samples during the EFSA baseline survey. However, please note that control material supplied by the EURL or generated using the method below will not work with all permissible primer/probe sets. If primer and probe sets different to those illustrated in Addendum 2 are used, it is the responsibility of the designated laboratory and the relevant NRL to ensure that EC RNA control material suitable for the primer/probe set selected is used.

Control plasmids as described in Addendum 5 are used for the production of EC RNA. These plasmids should be transformed and maintained in, and purified from, *E. coli* cells using standard molecular and microbiology techniques. Following purification of plasmid by e.g. commercial miniprep, a small amount should be linearised using a suitable restriction enzyme (to enable linearisation of the plasmid at a point shortly downstream of the target sequence) and buffers as recommended by the

manufacturer of the enzyme. For the plasmids used by the EURL, linearise using *Xba*I enzyme. The reaction should then be cleaned up using e.g. a commercial PCR purification kit.

For the plasmids used by the EURL, EC RNA can be in vitro transcribed using the SP6/T7 Riboprobe combination system (**Promega**, cat no. P1460[29]) as follows:

Add the following components at room temperature in the order listed:

5X transcription buffer	20 µl
100 mM DTT	10 µl
RNasin	2.5 µl
rATP, rGTP, rCTP, rUTP mix (2.5 mM each)	20 µl
linearised template DNA (max 1 µg/µl)	5 µl
T7 polymerase (for norovirus GI/GII EC RNA)	3 µl
water	39.5 µl

Mix by pipetting

Incubate for 2 h at 37ºC.

Add 5 µl RQ1 RNase-free DNase to the reaction.

Incubate for 15 mins at 37ºC.

The RNA should then be purified using RNA purification reagents (e.g. **QIAGEN** RNeasy Mini Kit [cat nos. 74103, 74104, 74106[30]] using the manufacturer's RNA cleanup protocol) and eluting in 100µl water.

The RNA preparation should be checked for freedom from significant contamination with DNA by assaying for target both with and without RT activity, for example, by assaying with both real-time RT-PCR mastermix where RT has been deactivated by heating at 95ºC, and untreated mastermix. If levels of DNA contamination higher than 0.1% are found, the preparation should be subjected to further treatment(s) with DNase.

The concentration of RNA can then be calculated using spectral absorption at 260 nm.

Multiplication of the A260 value by 4×10^{-8} (and by any dilution factor involved) will give the concentration of RNA in g/µl.

Divide this number by the mass in grams of a single EC RNA molecule to calculate the concentration of DNA in copies/µl (the mass of an individual RNA molecule is calculated by multiplying the RNA length in ribonucleotides by 320.5 (the molecular weight of an average ribonucleotide) and dividing by the Avogadro constant (6.02×10^{23}).

For the EC RNAs used by the EU-RL, the masses are as follows:

Norovirus GI	6.73×10^{-20} g	(126 b)
Norovirus GII	7.00×10^{-20} g	(131 b)

The preparation of RNA transcripts should then be diluted with a suitable buffer (e.g. TE buffer) (NOTE: Do not use water) to a concentration of approximately $1x10^4$–$1x10^5$ transcripts/µl, and frozen in single use aliquots.

[29] http://www.promega.com/catalog/country_select.asp?/default.asp&ckt=2
[30] http://www1.qiagen.com/SelectCountry.aspx

Appendix C – Examples of sample forms

Sample Form: Production Area Please check relevant grey shaded boxes and complete the green shaded boxes			
Sample reference number			
Country			
Production area code			
Production area name			
Classification at time of sampling	A		
	B		
	C		
	Fully classified		
	Seasonal		
	Preliminary		
Sampling Date and Time	Day XX/ Month XX/ Year XXXX - Hour XX		
Oyster Species	O. edulis		
	C. gigas		
	C. angulata		
Location of representative sampling point (WGS84 format)	Latitude	Longitude	
	Intertidal	Inshore	Offshore
Production system	Farmed	Wild	
	Raised Trestle		
	Suspended from sea-surface		
	Bottom-grown		
Remarks, any unusual conditions at time of sampling or deviations from sampling plan			
Sampler name			

Sample Form: Dispatch Centre Please check relevant grey shaded boxes and complete the green shaded boxes		
Sample reference number		
Country		
Dispatch Centre Approval Number		
Sampling Date and Time	Day XX/ Month XX/ Year XXXX - Hour XX	
Oyster Species	O. edulis	
	C. gigas	
	C. angulata	
Overall Batch Weight (Kgs)		
Production area 1:		
Production area code		
Production area name		
Classification at time of sampling	A	
	B	
	C	
Date of harvesting	Day XX/ Month XX/ Year XXXX	
Conditioning – duration in hours		
Relaying – duration in days		
Purification – duration in hours		
Indicate thermal status	Ambient	
	Actively heated	
	Actively cooled	
Temperature °C		
Oysters Origin	Wild	
Indicate country		
	Farmed	
Indicate fishing area code or name		
Production area 2		
Production area code		
Production area name		
Classification at time of sampling	A	
	B	
	C	

Date of harvesting	Day XX/ Month XX/ Year XXXX	
Conditioning – duration in hours		
Relaying – duration in days		
Purification – duration in hours		
Indicate thermal status	Ambient	
	Actively heated	
	Actively cooled	
Temperature °C		
Oysters Origin	Wild	
Indicate country		
	Farmed	
Indicate fishing area code or name		
Remarks, any unusual conditions at time of sampling or deviations from sampling plan		
Sampler name		

Appendix D – Supporting information

Appendix D can be found in the online version of this output.